An indirect immunofluorescence antibody test employing whole eggs as the antigen for the diagnosis of abdominal angiostrongyliasis

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Abdominal angiostrongyliasis is a potentially fatal zoonotic disease with a broad geographical distribution throughout Central and South America. This study assessed the performance of Angiostrongylus costaricensis *eggs as the antigen in an indirect immunofluorescence assay for the determination of parasite-specific IgG and IgG1 antibodies. For prevalence studies, an IgG antibody titre ≥ 16 was identified as the diagnostic threshold with the best performance, providing 93.7% sensitivity and 84.6% specificity. Cross reactivity was evaluated with 65 additional samples from patients with other known parasitic infections. Cross reactivity was observed only in samples from individuals infected with* Strongyloides stercoralis. *For clinical diagnosis, we recommend the determination of IgG only as a screening test. IgG1 determination may be used to increase the specificity of the results for patients with a positive screening test.*

Key words: abdominal angiostrongyliasis - immunodiagnosis - immunofluorescence - egg antigen

Abdominal angiostrongyliasis (AA) is a zoonotic infection with a broad geographical distribution throughout Central and South America. The aetiological agent is *Angiostrongylus costaricensis*, a nematode with an indirect life cycle that involves rodents as the definitive vertebrate hosts. The symptoms of human infections range from mild abdominal pain to an acute clinical presentation characterised by a strong inflammatory reaction in the intestinal wall and by the onset of non-specific symptoms, such as vomiting, anorexia, pain in the lower right quadrant, constipation and, occasionally, fever (Morera 1985, 1987, 1995, Graeff-Teixeira et al. 1991). Due to the non-specific symptoms of acute appendicitis syndrome, *A. costaricensis* is not usually considered as the primary aetiological agent until is it discovered with pathological examination.

AA remains a poorly studied helminthiasis in the majority of Latin America, except for within Brazil and Costa Rica. In several regions of Southern Brazil, reports have indicated that the prevalence of AA ranges from 2.8-28% (Graeff-Teixeira et al. 2005). In Costa Rica, an annual AA incidence of 20 cases per 100,000 subjects per year has been reported (Morera & Amador 1998) and it is thought that *A. costaricensis* is the causal agent in 20% of acute abdomen cases treated in the National Children's Hospital, as reported by the Medical Records and Statistics Department.

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Diagnosis in the clinical laboratory continues to be one of the greatest challenges in the management of AA. Because none of the stages of the parasite are shed in the faeces, a coproparasitological diagnosis of AA is impossible and the infection can only be confirmed in human beings by detection of the parasite in histological preparations obtained after a surgical intervention. To date, a latex agglutination test (Morera 1987, Morera & Amador 1998) and an enzyme-linked immunosorbent assay (ELISA) (Graeff-Teixeira et al. 1997, Geiger et al. 2001) have been used for the serological diagnosis of this disease. However, it has not been possible to standardise either of these two tests for their routine use in clinical laboratories. A recent study employed heterologous antigen from *Angiostrongylus cantonensis* adults in a serological test for the diagnosis of AA (Ben et al. 2010). The ELISA developed in that study showed higher sensitivity but lower specificity than the serological tests that are based on the *A. costaricensis* crude antigen.

Bender et al. (2003), in a study with human sera and using indirect immunofluorescence, reported that *A. costaricensis* eggs display high antigenicity and low cross-reactivity when used as the antigen. Furthermore, observations from experimental animal models have also shown the importance of eggs in the humoral response during an *A. costaricensis* infection (Geiger et al. 1999, Abrahams-Sandi et al. 2004, 2005). Recently, we conducted a study to assess the performance of *A. costaricensis* eggs as the antigen in an ELISA for the determination of parasite-specific IgG1 antibodies in human sera (Mesén-Ramírez et al. 2008). The specificity and sensitivity of the designed method were 87% and 90.5%, respectively. The main drawback of this technique was the high number of eggs required to prepare the antigen, which limits the number of assays that can be performed in the laboratory. With the objective of

solving this issue, and because an alternative method should have similar or better sensitivity and specificity compared to the ELISA method, we developed an indirect fluorescent antibody test (IFAT) similar to that used for *Toxoplasma gondii* employing whole *A. costaricensis* eggs as the antigen.

SUBJECTS, MATERIALS AND METHODS

Parasite maintenance - The parasites were maintained in the Medical Helminthological Laboratory of the Parasitology Department of Costa Rica University by perpetuating infections in *Sigmodon hispidus* and *Sarasinula plebeia*, which were used as definitive and intermediate hosts, respectively.

Antigen preparation - Whole eggs were collected as previously described by Abrahams-Sandi et al. (2005). Briefly, fertile female worms were incubated in Minimum Essential Medium Eagle (Sigma) supplemented with antibiotics at 37ºC, 5% CO_2 for 96 h. The culture medium was changed daily, pooled and centrifuged for 10 min at 1,000 *g* at 4ºC. The egg pellet was washed three times with phosphate buffered saline (PBS) supplemented with antibiotics and stored at -20ºC.

Serum samples - Serum samples obtained during the acute phase from 16 patients with a confirmed histopathological diagnosis of *A. costaricensis* (positive controls = C+) were kindly provided by Dr Pedro Morera (Pathology Service of Hospital San Juan de Dios, Costa Rica). A panel of 65 sera from patients with a positive faecal examination for at least one of the following parasites was provided by the Department of Parasitology, University of Costa Rica, and included as specificity controls: *Strongyloides stercoralis* (10), *Ascaris lumbricoides* (21), *Trichuris trichiura* (5), *Taenia* sp. (3) *Taenia saginata* (1), *Enterobius vermicularis* (2), *Ancylostoma/Necator* (6), *Entamoeba coli* (3) *Entamoeba histolytica/Entamoeba dispar* (6), *Giardia duodenalis* (4) and *Endolimax nana* (4). Twenty samples from healthy individuals living in an area with (10) or without (10) active *A. costaricensis* transmission and with a negative faecal examination were also kindly provided by the Department of Parasitology, University of Costa Rica, and included in the test as negative controls from an endemic zone (NCE) and normal negative sera (NS), respectively.

IFAT for A. costaricensis - *Slide preparation*: PTFE Printed Slides for immunofluorescence (10 or 12 well, 6 mm in diameter) were coated with 10 µL of whole egg suspension at a density of 10-15 egg/40X field and incubated overnight at room temperature (RT). The slides were washed three times in 1% PBS and allowed to dry at RT. Afterwards, the slides were immersed in acetone for 10 min at RT and allowed to dry. Fifteen microlitres of blocking solution [PBS 1%/bovine serum albumin (BSA) 5%] was added to each well and incubated for 15 min at RT. After washing as described, the slides were allowed to dry at RT and frozen at -20ºC until further use. Slides without significant morphological alterations in the eggs after fixation with acetone and storage at -20°C were used in the tests.

Determination of IgG and IgG1-specific antibody levels - The IFAT for *A. costaricensis* was carried out as described by Castro and Guerrero (2004) with some modifications. PTFE Printed Slides coated with antigen were removed from the freezer and allowed to thaw and dry thoroughly. Next, 15 µL of each test serum sample diluted from 1:8 to 1:1024 in PBS was added and incubated 45 min in a moist chamber at 37ºC. Afterwards, the slides were rinsed three times with PBS and 15 µL of 1:34 diluted fluorescein isothiocyanate (FITC)-conjugated anti-human IgG or IgG1 (Sigma) was added to each well and incubated 45 min as described above. Each FITC-conjugate for the detection of the two isotypes was diluted following manufacturer's specifications.

Finally the slides were washed and dried and a drop of PBS/glycerol/0.2% Evans blue (1:9, pH 9.2) was added. The light from the bound fluorescein-conjugated antibodies was detected with a Reichert Microstar IV fluorescence microscope. A positive reaction was indicated by intense homogeneous fluorescence in more than 50% of the eggs adsorbed on the slide after counting 30 microscopic fields.

RESULTS

Table I summarises the results of the study. Analysis of the C+ group showed a positive reaction for specific IgG antibodies in 15 of the 16 sera at a titre ≥ 8. For IgG1, 12 of these 16 sera showed a positive reaction at a titre ≥ 8. Among the group of 65 serum samples from patients with other known parasitic infections, cross-reactivity was observed only in those from people infected with *S. stercoralis* (10 serum samples) with an antibody titre of IgG ≤ 32. NCE and NS samples were negative in this test. The data regarding the sensitivity and specificity of the various serum antibody titres in the IFAT for *A. costaricensis* are shown in Table II.

TABLE I

Indirect immunofluorescence results of sera from patients with confirmed abdominal angiostrongyliasis

Serum sample identification	Reactivity IgG[a]	Reactivity IgG1[a]
1	64	32
2	16	negative
3	128	8
4	32	8
5	128	16
6	64	16
7	64	negative
8	64	negative
9	64	16
10	64	8
11	negative	negative
12	512	128
13	128	32
14	32	32
15	512	16
16	256	64

a: antibody titer.

DISCUSSION

Indirect immunofluorescence assays (such as indirect inclusion fluorescence antibody IIFA, IFA, IFAT, IFT) have been frequently used for the diagnosis of parasitic diseases. In one of the first such studies published, Welch and Dobson (1978) developed three immunofluorescent antibody assays for the study of infections by *Dirofilaria immitis*, *Toxocara canis* and *A. lumbricoides*. In that work, the authors demonstrated the advantage of using purified antigens for immunodiagnosis through techniques such as DFAT, IFAT, and CNBr-IFAT. Currently, IFAT is employed for the diagnosis of strongyloidiasis, fascioliasis, toxoplasmosis, giardiasis, Chagas disease, malaria and leishmaniasis (Wilson et al. 2006). By using either cryosections or complete forms from different developmental stages of helminthes or protozoans as antigens, the above-mentioned tests offer acceptable and satisfactory sensitivity and specificity.

Few studies have reported the use of parasite eggs as the antigen in an IFAT. *Ancylostoma/Necator*, *Toxocara* and *Schistosoma* are examples of parasites whose eggs have proven useful in the serological diagnosis of the disease (Zaman & Singh 1965, Glickman et al. 1978, Khalil et al. 1989, Alarcón de Noya et al. 2000). In 2003, Bender et al. (2003) described the high antigenicity of *A. costaricensis* eggs and, in 2008, Mesén-Ramírez et al. (2008) demonstrated the usefulness of a crude extract of this antigen in the development of an ELISA test. However, in the latter study, one of the main problems was the high number of eggs required to prepare the antigenic extract.

In an attempt to solve this issue, in the present work we employed *A. costaricensis* whole eggs as the antigen for the determination of specific IgG and IgG1 levels through an IFAT. The results obtained confirm the high antigenicity and low cross-reactivity of *A. costaricensis* eggs, which are important and necessary characteristics in an antigen employed in a diagnostic test, especially for diseases caused by helminthes. In agreement with a previous study (Mesén-Ramírez et al. 2008), cross-reaction was observed only against *S. stercoralis*, which suggests that common antigens exist in the eggs of these two parasites. A cross reaction against *S. stercoralis* was also observed by Graeff-Teixeira et al. (1997) in a previous study using adult forms of *A. costaricensis* as crude antigen; however, in that work cross-reactivity against other nematodes such as *A. lumbricoides* and *T. trichiura* was also reported.

According to our results, the highest specificity is obtained when determining the IgG1-specific isotype. For an IgG1 antibody titre ≥ 8, a specificity of 100% was achieved with a sensitivity of just 75%. However, the highest sensitivity of the IFAT designed in this study was obtained when determining total specific IgG against the parasite; in this case, for an IgG antibody titre ≥ 16, a sensitivity of 93.7% with a specificity of 84.6% was obtained.

Due to the cross-reactivity between different nematode antigens, intrinsic antigenic variation and the diversity in the immune response usually found in natural helminth infections, the ideal 100% sensitivity and specificity of an immunodiagnostic test is very difficult to achieve. In order to define the cut-off of a serological test, it is important to consider the clinical characteristics of the disease and the type of study in which the test is intended to be used. For example, it has been stated that for prevalence studies or for the diagnosis of a potentially fatal disease, the sensitivity of the diagnostic test is critical, whereas for the definition of cases for enrolment in a clinical trial, the optimal serological cut-off should correspond to the highest specificity, because false positives could lead to a misleading interpretation of the results. With respect to AA and taking into consideration the possibility of asymptomatic infections, for the determination of *A. costaricensis*-specific IgG through an IFAT, a diagnostic threshold of an antibody titre ≥ 16 should be established for prevalence studies.

With respect to clinical diagnosis, it is important to consider that AA may be fatal (especially in children less than 2 years old) and a false positive result on the diagnostic test may lead to unnecessary interventions. Thus, we recommend the determination of *A. costaricensis*-specific IgG only as a screening test. When a positive IgG result is

TABLE II

Estimated sensitivity and specificity of indirect fluorescent antibody test (IFAT) for *Angiostrongylus costaricensis* at different serum antibody titers

	IgG antibody		IgG1 antibody	
Titer	Sensitivity[a] %	Specificity[b] %	Sensitivity[a] %	Specificity[b] %
≥ 8	93.8 (15/16)	84.6 (55/65)	75 (12/16)	100 (65/65)
≥ 16	93.8 (15/16)	84.6 (55/65)	56.2 (9/16)	100
≥ 32	87.5 (14/16)	87.7 (57/65)	31.5 (5/16)	100
≥ 64	75 (12/16)	100 (65/65)	12.5 (2/16)	100
≥ 128	37.5 (6/16)	100	6.25 (1/16)	100
≥ 256	18.8 (3/16)	100	0 (0/16)	100
≥ 512	12.5 (2/16)	100	0	100
≥ 1.024	0 (0/16)	100	0	100

a: number of IFAT positive control (C+)/total C+; *b*: number of IFAT negative specificity control (SC)/total SC.

obtained, the subsequent determination of IgG1 could be helpful to complement the diagnosis of AA; however, as described for other helminthiases, serological tests should be accompanied by clinical criteria for a correct diagnosis.

In order to improve the specificity of tests that employ eggs as antigen, some authors have proposed the preadsorption of serum samples with eggs of the problem species (Cypess et al. 1977, Pollard et al. 1979). Additionally, for *Schistosoma*, Alarcón de Noya et al. (2000) suggest eliminating the carbohydrates responsible for the cross reactivity by treating the antigen with sodium metaperiodate. We have not yet employed either of these methods to try to eliminate the cross reaction with *S. stercoralis*. However, in clinical cases, a parallel coproparasitological analysis could be performed to rule out infection by *S. stercoralis* because, in contrast with AA, a patient with this nematode is usually capable of shedding parasite larvae in faeces.

Compared to the ELISA designed by us (Mesén-Ramírez et al. 2008), the IFAT described in the present paper has the advantage of providing a quantitative result through the precise determination of the specific antibody titre. Additionally, the number of eggs required to prepare the reaction microslides is lower, which can help laboratories analyse a higher number of serum samples. Furthermore, the sensitivity and specificity of this IFAT for *A. costaricensis* is comparable or even better than that of any of the techniques published to date for this disease (Geiger et al. 2001, Mesén-Ramírez et al. 2008). Further studies are necessary to evaluate the usefulness of this IFAT for the serological follow-up of AA patients and to determine seroreversion or decreases in antibody titres after clinical recovery or surgical intervention.

We suggest the determination of specific IgG antibody levels as a screening test for suspected clinical AA cases and IgG1 levels should subsequently be determined to obtain greater specificity in the case of a positive IgG result.

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REFERENCES

Abrahams-Sandi E, Geiger SM, Fernández-Quesada K, Schulz-Key H 2005. Specific antibody production against different life cycle stages during an experimental *A. costaricensis* infection in mice. *Rev Biomed 16*: 239-246.

Abrahams-Sandi E, Hoffmann WH, Graeff-Teixeira C, Schulz-Key H, Geiger SM 2004. Long-term observations on mouse strains experimentally infected with *Angiostrongylus costaricensis*. *Parasitol Res 93*: 230-234.

Alarcón de Noya B, Colmenares C, Lanz H, Caracciolo MA, Losada S, Noya O 2000. *Schistosoma mansoni*: immunodiagnosis is improved by sodium metaperiodate which reduces cross-reactivity due to glycosylated epitopes of soluble egg antigen. *Exp Parasitol 95*: 106-112.

Ben R, Rodrigues R, Agostini AA, Graeff-Teixeira C 2010. Use of heterologous antigens for the immunodiagnosis of abdominal angiostrongyliasis by an enzyme-linked immunosorbent assay. *Mem Inst Oswaldo Cruz 105*: 914-917.

Bender AL, Maurer RL, Silva MCF, Ben R, Terraciano TB, Aramburu da Silva AC, Graeff-Teixeira C 2003. Ovos e órgãos reprodutores de fêmeas de *Angiostrongylus costaricensis* são reconhecidos mais intensamente por soros humanos de fase aguda na angiostrongilíase abdominal. *Rev Soc Bras Med Trop 36*: 449-454.

Castro CA, Guerrero BO 2004. *Técnicas de diagnóstico parasitológico*, 1st ed., Universidad de Costa Rica, San José, 113 pp.

Cypess RH, Karol MH, Zidian JL, Glickman LT, Gitlin D 1977. Larva-specific antibodies in patients with visceral larva migrans. *J Infect Dis 135*: 633-640.

Geiger SM, Graeff-Teixeira C, Soboslay PT, Schulz-Key H 1999. Experimental *Angiostrongylus costaricensis* infection in mice: immunoglobulin isotype responses and parasite-specific antigen recognition after primary low-dose infection. *Parasitol Res 85*: 200-205.

Geiger SM, Laitano AC, Sievers-Tostes C, Agostini AA, Schulz-Key H, Graeff-Teixeira C 2001. Detection of the acute phase of abdominal angiostrongyliasis with a parasite-specific IgG enzyme linked immunosorbent assay. *Mem Inst Oswaldo Cruz 96*: 515-518.

Glickman L, Schantz P, Dombroske R, Cypess R 1978. Evaluation of serodiagnostic tests for visceral larva migrans. *Am J Trop Med Hyg 27*: 492-498.

Graeff-Teixeira C, Agostini AA, Camillo-Coura L, Ferreira-da-Cruz MF 1997. Seroepidemiology of abdominal angiostrongyliasis: the standardization of an immunoenzymatic assay and prevalence of antibodies in two localities in Southern Brazil. *Trop Med Int Health 2*: 254-260.

Graeff-Teixeira C, Camillo-Coura L, Lenzi HL 1991. Angiostrongiliase abdominal: nova parasitose no sul do Brasil. *Rev Ass Med Rio Gr Sul 35*: 91-98.

Graeff-Teixeira C, Goulart AH, Brum Cde O, Laitano AC, Sievers-Tostes C, Zanini GM, Bered PL, Morassutti A, Geiger S, Abrahms-Sandi E, Oliveira FT, Maurer RL, Aguiar LF, Garrido CT, da Silva AC, Rodriguez R, Schulz-Key H, Agostini AA 2005. Longitudinal clinical and serological survey of abdominal angiostrongyliasis in Guaporé, Southern Brazil, from 1995 to 1999. *Rev Soc Bras Med Trop 38*: 310-315.

Khalil HM, Azab ME, Safar EH, Bebars MA, el-Hady HM, Khattab HM 1989. Immunodiagnostics of visceral toxocariasis. *J Egypt Soc Parasitol 19*: 381-393.

Mesén-Ramírez P, Abrahams-Sandí E, Fernández-Quesada K, Morera P 2008. *Angiostrongylus costaricensis* egg antigen for the immunodiagnosis of abdominal angiostrongyliasis. *J Helminthol 82*: 251-254.

Morera P 1985. Abdominal angiostrongyliasis: a problem of public health. *Parasitol Today 1*: 173-175.

Morera P 1987. Angiostrongiliasis abdominal: un problema de salud pública? *Rev Asoc Guatemalteca Parasitol Med Trop 2*: 9-11.

Morera P 1995. Abdominal angiostrongyliasis. In P Morera (ed.), *Enteric infection: intestinal helminths*, Chapman & Hall, London, p. 225-230.

Morera P, Amador JA 1998. Prevalencia de la angiostrongilosis abdominal y la distribución estacional de la precipitación. *Rev Costarric Salud Publica 7*: 1-14.

Pollard ZF, Jarrett WH, Hagler WS, Allain DS, Schantz PM 1979. ELISA for diagnosis of ocular toxocariasis. *Ophthalmology 86*: 743-752.

Welch JS, Dobson C 1978. Immunodiagnosis of parasitic zoonoses: comparative efficacy of three immunofluorescence tests using antigens purified by affinity chromatography. *Trans R Soc Trop Med Hyg 72*: 282-288.

Wilson M, Schantz P, Nutman T 2006. Molecular and immunological approaches to the diagnosis of parasitic infections. In B Detrick, RG Hamilton, JD Folds, *Manual of molecular and clinical laboratory immunology*, ASM Press, Washington, p. 557-568.

Zaman V, Singh M 1965. Immuno-fluorescent studies with hookworms I. Antigenic relationships of ova. *Trans R Soc Trop Med Hyg 59*: 690-693.