# Detection of the Acute Phase of Abdominal Angiostrongyliasis with a Parasite-specific IgG Enzyme Linked Immunosorbent Assay

**Stefan Michael Geiger/*/***, Antônio Carlo Laitano, Charlotte Sievers-Tostes, Aventino Alfredo Agostini/**, Hartwig Schulz-Key/***, Carlos Graeff-Teixeira/+**

Laboratório de Parasitologia Molecular, Instituto de Pesquisas Biomédicas e Laboratório de Biologia Parasitária, Faculdade de Biociências, PUCRS, Av. Ipiranga 6690, 90610-000 Porto Alegre, RS, Brasil *Centro de Pesquisas René Rachou-Fiocruz, Belo Horizonte, MG, Brasil **Instituto de Patologia de Passo Fundo, Passo Fundo, RS, Brasil ***Institut für Tropenmedizin, Eberhard Karls Universität, Tübingen, Germany

Angiostrongylus costaricensis *may cause intestinal lesions of varied severity when it accidentally infects man in Central and South America. First-stage larvae have never been detected in stools. Therefore, a parasite-specific IgG ELISA was evaluated for the determination of the acute phase of infection. The specificity and the sensitivity of the immunoassay was shown to be 76.2% and 91.1%, respectively. Eight serum samples taken from patients with histopathological diagnosis, at different time points (3 to 15 months) after surgical treatment, showed a sharp and early decline in antibody reactivity. The titration of anti-*A. costaricensis *antibodies has proved to be a useful method for the diagnosis of acute abdominal angiostrongyliasis.*

Key words: abdominal angiostrongyliasis - serology - eosinophilic gastroenteritis - immunodiagnosis

*Angiostrongylus costaricensis* is a parasitic nematode of rodents, with a widespread occurrence in the Americas: from the southern United States to northern Argentina (Ubelaker & Hall 1979, Demo & Pessat 1986). The intermediate hosts are molluscs, especially slugs from the Veronicellidae family (Morera 1973). The human infection is established through the ingestion of third-stage larvae (L3) when water or food is contaminated with the mucous secretions eliminated by the intermediate host or when a small mollusc is accidentally ingested. Acute abdominal disease may develop with severe inflammatory and thrombotic lesions in the intestines (Céspedes et al. 1967).

Inflammation in the intestinal wall probably prevents the elimination of larvae in faeces and the diagnosis relies upon histopathological examination of surgical specimens obtained in the most severe cases when surgical treatment for the correction of intestinal perforations or obstruction is required (Graeff-Teixeira et al. 1991).

We report the evaluation of an immunoenzymatic test for the detection of the acute phase of abdominal angiostrongyliasis.

## MATERIALS AND METHODS

Serum samples of patients with histopathological diagnosis (positive controls = +C) have been collected for the last 15 years in the southernmost states of Brazil. For the initial testing of class- and subclass-specific serological reactivity, nine +C sera from the time of surgery (time "zero") and from 6 to 15 months after surgery were evaluated. A panel of 45 serum samples from patients with positive fecal examination for at least one of the following parasites was included as specificity controls (SC): *Ascaris lumbricoides*, hookworm, *Entamoeba coli*, *Giardia duodenalis*, *Schistosoma mansoni*, *Strongyloides stercoralis*, and *Trichuris trichiura*. These patients were living in urban areas of Rio de Janeiro, São Paulo and Recife, outside the known endemic area for abdominal angiostrongyliasis. Samples from patients infected with *Wuchereria bancrofti* were kindly provided by Dr Gerusa Dreyer (Fiocruz, Recife, Brazil). Sera (n=20) from healthy students (without fever, abdominal pain or any other evidence of systemic diseases) at the Pontifícia Universidade Católica do Rio Grande do Sul were also included in the testing as "normal negative sera".

This work was supported by PUCRS, Fapergs (97.0476.0), Fortüne Program from the University of Tübingen and Probral Program (055/97 Capes, Brasil and DAAD, Germany)
+Corresponding author and recipient of a CNPq PQ IIA fellowship. Fax: +55-51-320.3312
E-mail: graeffteixeira@netscape.net
Received 14 August 2000
Accepted 14 November 2000

*Antigen preparation* - The recovery of worms (Santa Rosa strain) from experimentally infected rodents is described elsewhere (Graeff-Teixeira et al. 1997). About 70 female worms were homogenized 4x30 sec at 4°C in a Tekmar tissue homogenizer in a total volume of 3 ml of Tris-HCl-buffer containing the following protease inhibitors: 1 mM PMSF (phenylmethylsulphonylflouride, SIGMA), 1 mM TLCK (Na-p-tosyl-L-lysine-cloromethyl ketone, SIGMA) and 1 mM EDTA (ethylenediamine-tetra-acetic acide, SIGMA). The suspension was centrifuged twice at 12,000 xg for 20 min at 4°C and the resulting soluble supernatant was stored at -20°C until further use. The protein concentration was determined by the comparison of UV absorbances at 280 and 260 nm (Harlow & Lane 1988).

Polystyrene plates were coated with 100 µl of a 7 µg/ml antigen solution in a 0.06 M carbonate-bicarbonate buffer, pH 9.6 and blocked in a PBS 0.05% Tween-20 solution with 5% skimmed milk for 3 h at room temperature (RT). Test sera diluted 1:500 in PBS were incubated for 2 h at RT. Anti-IgG or IgE peroxidase-conjugated antibodies (1:1000, SIGMA), or IgG1 (1:2000, SIGMA) or IgG4 (1:1000, SIGMA) were used as secondary antibodies and incubated for 2 h at RT. For the anti-subclasses biotinylated antibodies, an intermediate incubation step with avidine-peroxidase (1:1000, SIGMA) for 90 min at RT (1:2000 in blocking buffer) was performed. For development, 0.04% OPD (o-phenylenediamine, SIGMA) in 0.012% H2O2/citrate-phosphate buffer, pH 5 was added and the optical density (OD) was measured at 492 nm in an automated reader (Behring, EL311 Microplate Reader).

## RESULTS

Confirming previous results with ELISA and immunoblotting, IgG1 was shown to be the dominant IgG response and the values for parasite-specific IgG4 and IgE antibodies were low or not detectable, even with acute phase sera highly reactive for total IgG (data not shown). Some of the +C presented a very low reactivity, demonstrating the diversity usually found in natural infections (Fig. 1).

The OD cutoff value of 0.165 resulted from the sum of mean OD (0.06) plus two standard deviation (SD=0.05) of six SC sera tested at 1:500 dilution, in a preliminar experiment. An extended analysis of 45 SC sera confirmed the adequacy of that OD cutoff value, with a mean OD of 0.05 (SD = 0.06).

Specificity of the IgG-ELISA was estimated to be 91.1% (41 out of 45 SC were negative), while sensitivity was 76.2% (16 out of 20 +C were reactive) (Fig. 1).

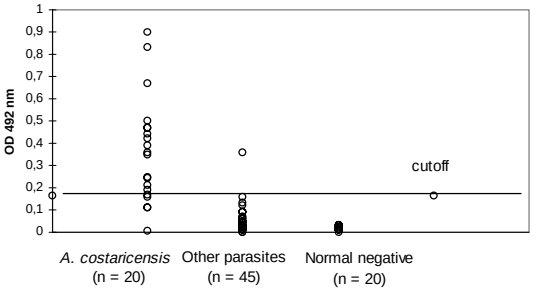


Fig.1: parasite-specific IgG-reactivity in sera from patients with confirmed *Angiostrongylus costaricensis* infection (positive-controls), patients with other parasitic infections (specificity-controls) and normal negative controls.

The maximum positive titer for specific IgG antibodies in sera from selected *A. costaricensis* patients ranged between a 1:500 and a 1:8000 dilution, whereas the specificity control sera reached maximum titers of 1:250 (Table I). Furthermore, as shown in Table II and Fig. 2, *A. costaricensis* patients showed a prominent decrease in specific IgG antibodies after surgical intervention, i.e. post-acute-phase.

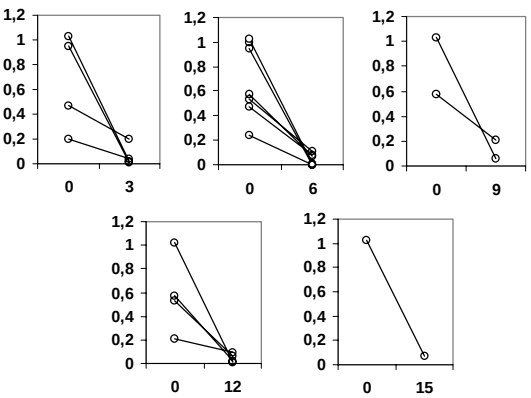


Fig. 2: reduction in parasite-specific IgG-reactivity (y axis, optical density 490 nm) in serum samples from eight *Angiostrongylus costaricensis*-infected patients at 3, 6, 9 and 15 months ("x" axis) after the acute phase. Samples were not available from all time points and only one patient was followed for 15 months.

## DISCUSSION

There is a possibility that first-stage larvae from *A. costaricensis* is eliminated in human faeces, but trials to detect them have been unsucessful. Therefore, the diagnosis of *A. costaricensis* infection depends on anatomopathological examination of surgical specimens. An ELISA for seroepidemiology was previously standardized but its value for discriminating the acute phase of the disease was unknown (Graeff-Teixeira et al. 1997).

TABLE I

Reactivity and titration of acute phase sera with the IgG-ELISA for *Angiostrongylus costaricensis*

| Serum register | Maximal positive titer | 1:250 | 1:500 | 1:1000 | 1:2000 | 1:4000 | 1:8000 |
|---|---|---|---|---|---|---|---|
| *A. costaricensis* control sera | | | | | | | |
| 273 | 1:2000 | + | + | + | + | NR | NR |
| 276 | 1:2000 | + | + | + | + | NR | NR |
| 303 | 1:8000 | + | + | + | + | + | + |
| 305 | 1:4000 | + | + | + | + | + | NR |
| 312 | 1:500 | + | + | NR | NR | NR | NR |
| 317 | 1:8000 | + | + | + | + | + | + |
| 307 | 1:2000 | + | + | + | + | NR | NR |
| 266 | 1:1000 | + | + | + | NR | NR | NR |
| Specificity control sera | | | | | | | |
| 172 | NR | NR | NR | NR | NR | NR | NR |
| 216 | 1:250 | + | NR | NR | NR | NR | NR |
| 214 | NR | NR | NR | NR | NR | NR | NR |
| 220 | 1:250 | + | NR | NR | NR | NR | NR |
| 204 | NR | NR | NR | NR | NR | NR | NR |
| 210 | NR | NR | NR | NR | NR | NR | NR |

NR: non reactive

TABLE II

Reactivity (maximum titer) of sera from patients with confirmed abdominal angiostrongyliasis, at several periods post-acute-phase

| Patients | Acute phase | 3 months | 6 months | 9 months | 12 months | 15 months |
|---|---|---|---|---|---|---|
| 273 | 1:2000 | 1:500 | NR | | | |
| 313 | 1:2000 | | | NR | | |
| 303 | 1:8000 | NR | NR | | NR | NR |
| 312 | 1:500 | | | NR | NR | |
| 305 | 1:4000 | | NR | | NR | |
| 317 | 1:8000 | NR | NR | | | |
| 307 | 1:2000 | | NR | | NR | |
| 266 | 1:1000 | | NR | | | |

NR: non reactive; samples were not available at every time point.

A panel of positive control sera collected at different time points after surgery made the present evaluation of an ELISA system for diagnosis of the acute phase of infection possible. Acute phase may be defined in abdominal angiostrongyliasis as the period when worms are alive, reproduce, and larval stages are shed into the intestinal layers for some time up to the complete removal of their bodies. From an ongoing longitudinal study in southern Brazil, there are indications that disease manifests by acute abdominal pain episodes of short duration (2-3 days), sometimes recurrent in several episodes over 6-8 months. Perforation of the intestinal wall and severe inflammatory reactions may eventually lead to surgical intervention, but probably a complete and spontaneous remission is the norm.

As already known for other helminth infections, e.g. schistosomiasis or filarial infections (Garate et al.1990, Hamilton et al.1998, Harnett et al.1998), crude antigenic preparations are generally problematic for immunodiagnosis because of their broad cross-reactivity with other helminth species (Parkhouse & Harrison 1989, Lynch et al. 1998). Because purified specific antigenic components from *A. costaricensis* are not available, a crude female worm antigen preparation has been employed for antibody detection in the present evaluation. Several attempts have failed (results not shown) to identify acute phase-specific reactivity to defined antigenic components, including subclasses and isotypic analysis: IgE, IgG1, IgG2, IgG3, IgG4 and IgM. IgG3, IgG4, and IgE were

also tested in ELISA, without improvement of specificity. Tests for detection of IgA are underway.

The definition of the cutoff value in immunodiagnosis of abdominal angiostrongyliasis must take into consideration the possibility that asymptomatic infections occur in the endemic area, preventing the identification of "normal endemic controls". With the development of a nucleic acid detection method (unpublished observations) it will be possible to define the "normal endemic controls" as healthy individuals negative to abdominal angiostrongyliasis by polymerase chain reaction. Ideally, cross-reactivity is reduced by selecting purified antigens specific to the parasite, studying humoral responses at the isotype level, or removing cross-reacting antibodies by absorption of sera with heterologous antigens. The ideal of a 100% sensitive and specific immunodiagnostic test is hard to achieve, since trials to improve specificity usually lead to lower sensitivity, and vice-versa. For prevalence studies, usually in asymptomatic or chronically infected populations and when the immunological test stands alone to establish the diagnosis, sensitivity is critical. Error occurs in a population based approach. For clinical diagnosis, sensitivity is not as critical as specificity, since several criteria are considered simultaneously and data from serological tests usually are not to be used alone. Specificity is critical in this situation, because a positive result may lead to intervention procedures and error occurrs on individual basis. For this reason, in the present analysis, the cutoff value was established from the reactivity found in individuals with heterologous infections, living in an area supposedly free from transmission of *A. costaricensis*.

Previous unpublished data from western-blot analysis had indicated that humoral response decreased sharply in the first month after the acute episode leading to surgery and histopathological diagnosis of abdominal angiostrongyliasis. This has been confirmed by the present semi-quantitative analysis of serum antibodies, either expressed by the optical density (Fig. 2) or by their titer at dilution assays (Tables I, II). As humans are accidental hosts for *A. costaricensis*, they react with a strong inflammatory reaction in the intestinal and mesenteric tissues (Graeff-Teixeira et al. 1991). The sharp declining in the humoral immune response may indicate that the parasite does not survive for a long time and antigenic stimulation rapidly terminates. Therefore, this infection does not persist into a chronic phase and seroepidemiological studies looking for past infections would face huge difficulties to detect specific long-term humoral reactivity.

Apart from the poor performance of the ELISA system using crude antigen, it is clearly demonstrated that semi-quantitative antibody detection methods can diagnose the acute phase of infection in abdominal angiostrongyliasis.

## REFERENCES

Céspedes R, Salas J, Mekbel S, Troper L, Mullner F, Morera P 1967. Granuloma entéricos y linfáticos con intensa eosinofilia tisular producidos por um estrongilídeo (Strongylata). *Acta Med Cost 10*: 235-255.

Demo OJ, Pessat OAN 1986. Angiostrongilosis abdominal. Primer caso humano encontrado em Argentina. *Prensa Med Argentina 73*: 732-738.

Garate T, Kliks MM, Cabrera Z, Parkhouse RME 1990. Specific and cross-reacting antibodies in human response to *Onchocerca volvulus* and *Dracunculus medinensis* infections. *Am J Trop Med Hyg 42*: 140-147.

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 Internat Health 2*: 254-260.

Graeff-Teixeira C, Camillo-Coura L, Lenzi HL 1991. Histopathological criteria for diagnosis of abdominal angiostrongyliasis. *Parasitol Res* 77: 606-611.

Hamilton JV, Klinkert M, Doenhoff MJ 1998. Diagnosis of schistosomiasis: antibody detection, with notes on parasitological and antigen detection methods. *Parasitology 117*: S41-S57.

Harlow E, Lane D 1988. *Antibodies – A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 726 pp.

Harnett W, Bradley JE, Garate T 1998. Molecular and immunodiagnosis of human filarial nematode infections. *Parasitology 117*: S59-S71.

Lynch NR, Wilkes LK, Hodgen AN, Turner KJ 1988. Specificity of *Toxocara* ELISA in tropical populations. *Parasite Immunol 10*: 323-337.

Morera P 1973. Life history and redescription of *Angiostrongylus costaricensis* Morera and Céspedes, 1971. *Am J Trop Med Hyg 22*: 613-621.

Parkhouse RME, Harrison LJS 1989. Antigens of parasitic helminths in diagnosis, protection and pathology. *Parasitology 99*: S5-S19.

Ubelaker JE, Hall NM 1979. First report of *Angiostrongylus costaricensis* Morera and Céspedes, 1971 in the United States. *J Parasitol 65*: 307.