Use of heterologous antigens for the immunodiagnosis of abdominal angiostrongyliasis by an enzyme-linked immunosorbent assay

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Angiostrongylus costaricensis has a broad geographic distribution spanning from North to South America and the infections of vertebrates with this nematode can result in abdominal complications. Human infections are diagnosed by histological or serological methods because the isolation of larvae from feces is not feasible, as most parasites become trapped in intestinal tissues due to intense eosinophilic inflammation. Because A. costaricensis is difficult to maintain in the laboratory, an immunodiagnostic IgG enzyme-linked immunosorbent assay (ELISA) using antigens from the congeneric Angiostrongylus cantonensis species was evaluated against a panel of serum samples from patients who were histologically diagnosed with A. costaricensis infections. Sera from uninfected individuals and individuals infected with other parasites were used as controls. The sensitivity and specificity of the assay were estimated at 88.4% and 78.7%, respectively. Because the use of purified or cloned antigens has not been established as a reliable diagnostic tool, the use of heterologous antigens may provide a viable alternative for the development of an ELISA-based immunodetection system for the diagnosis of abdominal angiostrongyliasis.

Key words: abdominal angiostrongyliasis - Angiostrongylus costaricensis - Angiostrongylus cantonensis - immunodiagnosis - eosinophilic gastroenteritis - helminths

Human abdominal angiostrongyliasis (AA) is caused by infection by the intra-arterial nematode Angiostrongylus costaricensis that complete their sexual cycle in rodents. Humans are accidental hosts and the localization of adult worms inside mesenteric arteries leads to the development of inflammatory lesions (Graeff-Teixeira et al. 1991). AA has been diagnosed across Central and South America and isolated imported cases have been reported in Europe and the United States. Molecular diagnostic methods are important for diagnosing human disease because first-stage larvae can only be detected microscopically in the feces of the definitive host, rodents (Pena et al. 1995).

Angiostrongylus cantonensis is a metstrongylid nematode with a broad geographic distribution spanning from Asia, the Pacific Islands, Central America and more recently, South America. Adult worms live within the pulmonary arteries of definitive hosts (e.g. the black rat, Rattus norvegicus) following the maturation of late larval stages in the central nervous system. Accidental infections of humans may result in eosinophilic meningitis due to the inability of larvae to exit meningeal tissues (Wang et al. 2008).

The utilization of antigens for the immunodiagnosis of AA has been hindered by complications that are associated with maintaining the life cycle of A. costaricensis in the laboratory for use as a source of crude antigen. However, A. cantonensis can be maintained and propagated in the laboratory more effectively and can therefore provide large amounts of heterologous antigens; these antigens can be used to develop immunodiagnostic assays that detect cross-reactive A. costaricensis antibodies. This study evaluated the use of A. cantonensis antigens in a standardized enzyme-linked immunosorbent assay (ELISA) for the diagnosis of A. costaricensis infections.

SUBJECTS, MATERIALS AND METHODS

The parasite - A. cantonensis was kindly provided by Professor Kentaro Yoshimura, Akita University Medical School, Japan. The parasite was maintained in the Parasitology Biology Laboratory of Pontifical Catholic University of Rio Grande do Sul (PUCRS), by perpetuating the infectious cycle in R. norvegicus and Biomphalaria glabrata that are used as definitive and intermediate hosts, respectively.

Animal handling - Animal experiments were carried out according to regulations that were established by the Brazilian College of Animal Experimentation and the protocol was approved by the University Ethical Committee for research involving human subjects.

Sera - Samples (n = 26) from patients with histopathological diagnoses of A. costaricensis infections were used as positive controls (PC) (Graeff-Teixeira et al. 1991). These patients came from the states of RS, Santa Catarina and Paraná and they were diagnosed after surgical treatment for complicated courses of abdominal disease. Serum samples were collected from 1-8 weeks after the beginning of symptoms. A panel of 47 serum samples from patients with positive fecal diagnoses for at least one of the following parasites were included as specificity con-
controls: *Ascaris lumbricoides*, hookworm, *Entamoeba coli*, *Giardia duodenalis*, *Schistosoma mansoni*, *Strongyloides stercoralis* and *Trichuris trichiura*. These patients lived in the urban areas of Rio de Janeiro, São Paulo or Recife and they were outside of the areas that are endemic for AA. Sera (n = 11) from healthy students (without fever, abdominal pain or any other signs of systemic diseases) from PUCRS were also included as negative controls (NC).

**Antigen preparation** - Female *A. cantonensis* worms were removed from the pulmonary arteries and cardiac cavities of infected rats, washed in saline and stored at -20°C until use. For antigen preparation, 60 worms were frozen in liquid nitrogen and homogenized until they were reduced to a fine powder. For protein extraction, 1 mL of 20-mM NaCl Tris-HCl containing 1-mM phenylmethylsulphonyl-flouride (Sigma, St. Louis, MO), 1-mM Na-p-tosyl-L-lysine chloromethylketone TLCK (Sigma) and 1-mM ethylene-diamine-tetra-acetic acid EDTA (Sigma) were added and the solution was sonicated three times for 2 min at 30% amplitude. The suspension was centrifuged twice at 12,000 g for 20 min at 4°C and the resulting soluble supernatant was stored at -20°C until use. The protein concentrations were determined using the Bradford assay (Bradford 1976).

**ELISA** - The optimal antigen concentrations and serum dilutions for ELISAs were determined by titration and the optimal concentration of crude antigen was determined to be 5 µg/mL. The assays were carried out by coating polystyrene plates with 100 µL of 5-µg/mL antigen in coating buffer (15-mM sodium carbonate and 35-mM sodium bicarbonate, pH 9.6) at 4°C for 12 h overnight. After three washes with phosphate buffered saline (PBS)/0.05% Tween-20, the plates were blocked with 5% non-fat milk in 0.05% Tween-20/PBS solution for 3 h at room temperature (RT). The test sera (1:200) and peroxidase-conjugated goat anti-human IgG antibodies (1:1000, Zymed, USA) were diluted in blocking buffer. The antigen-coated wells were incubated with serum samples and the secondary antibodies were incubated for 2 h at RT. Binding was colorimetrically visualized following the addition of 0.04% o-phenylenediamine (Sigma) tablets that were dissolved in 0.012% H₂O₂/citrate-phosphate buffer (pH 5) for 15 min in the dark at RT. HCl (2N, 100 µL) was added to the wells to stop the reaction and the optical density (OD) was measured at 490 nm. Cut-off values were calculated as the average OD readings plus two standard deviations.

**RESULTS**

An *A. cantonensis* crude antigen-based ELISA was developed to diagnose *A. costaricensis* infections. The sensitivity and specificity of the test were determined by comparing OD readings from the sera of *A. costaricensis*-infected patients to the sera of uninfected controls or patients who were infected with different parasites (Table). Twenty-three out of 26 PC sera had positive OD readings compared to 10 out of 47 sera from patients with other parasitic infections, resulting in a sensitivity of 88.4% and a specificity of 78.7% (Figure, Table). When the cut-off values were calculated from NC absorbance readings, the sensitivity and specificity were 76.9% and 93.6% (Table).

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Sensitivity and specificity of an <em>Angiostrongylus cantonensis</em> enzyme-linked immunosorbent assay (ELISA) for the detection of IgG anti-<em>Angiostrongylus costaricensis</em> antibodies</th>
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<td>Total</td>
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<td>Cut-off: medium optical density + 2 standard deviation* of normal control sera</td>
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<td>Cut-off: medium optical density + 2 standard deviation of specificity control sera</td>
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*a*: optical density at 490 nm.
DISCUSSION

_A. costaricensis_ crude antigens have been used for the immunodiagnostics of angiostrongyliasis since 1997 and later in the diagnosis of acute angiostrongyliasis (Graeff-Teixeira et al. 1997, Geiger et al. 2001). Previously used antibody-based detection systems included precipitation reactions (Sauerbrei 1977) and latex agglutination using crude antigenic preparations; the latter has been used for many years in Costa Rica (Kramer et al. 1998). In addition, low molecular weight peptides (Abraham et al. 2004), egg antigens (Mesén-Ramírez et al. 2008) and crude excretion-secretion antigens, as well as host antibody response at both the class (IgE, IgA) and isotype (IgG1, IgG2, IgG3, IgG4) (Geiger et al. 2001) levels, have been studied but not extensively evaluated in clinical or epidemiological settings. Due to insufficient sensitivities, specificities and reproducibilities of these assays in diagnosing _A. costaricensis_ infections and the unavailability of protocols utilizing purified or recombinant antigens for diagnostic purposes, improvements to currently available crude antigen-based ELISA assays are necessary.

Because _A. cantonensis_ and _A. costaricensis_ are congeneric, these organisms likely share various antigenic determinants (Dekumyoy et al. 2000). Furthermore, _A. cantonensis_ can be easily propagated in the laboratory and can thus provide substantial amounts of crude antigen for use in ELISAs. The feasibility of this congeneric species approach has already been demonstrated by the diagnoses of patients who are infected with parasites that share epitopes, such as _S. stercoralis_ and _Strongyloides venezuelensis_ (Feliciano et al. 2010) or _Strongyloides ratti_ (Rodrigues et al. 2007). This approach has been particularly promising in the development of alternative methods for the diagnosis of cisticercosis, strongyloidiasis and hidatidosis (Vaz et al. 1997, Sako et al. 2006, Feliciano et al. 2010, Oliveira et al. 2010, da S Ribeiro et al. 2010). In addition, Dekumyoy et al. (2000) studied the use of a crude antigen preparation that was derived from _A. costaricensis_ as a heterologous target for the detection of antibodies in patients with eosinophilic meningitis.

In addition to a potential use for diagnosis, heterologous antigens may also be useful for vaccination strategies that are based on the elicitation of cross-protective immunity (Stropkovská et al. 2010) and the modulation of the immune response (Page et al. 2006). Cross-reactivity or molecular mimicry may elicit the suppression or activation of immune responses that are associated with the pathogenesis of infectious diseases (Kierszenbaum 2003, Gironès et al. 2005, Millington et al. 2006).

The importance of developing molecular diagnostic methods or immunodiagnostic techniques for the diagnosis of human infections with _A. costaricensis_ is highlighted by the fact that human cases are difficult to diagnose due to the challenge in identifying trapped larvae (Graeff-Teixeira et al. 1991). For example, a single larva was identified histologically in transit between necrotic intestinal mucosal tissues and the lumen (unpublished observations). More importantly, neither larvae nor eggs have ever been detected in human stool samples and the examination of feces is not routinely performed in the diagnosis of AA.

Compared to a previous report on ELISA using _A. costaricensis_ antigen (Geiger et al. 2001), the ELISA using _A. cantonensis_ crude antigen preparation in this study showed increased sensitivity (76% in the previous study vs. 88.4% in this study) and decreased specificity (91.1% in the previous study vs. 78.7% in this study). Purified or cloned antigens have not been established as reliable diagnostic tools. Therefore, the use of heterologous antigens may provide a viable alternative for developing an ELISA-based immunodetection system for use in the diagnosis of AA.

REFERENCES


