

# The Current Status of Laboratory Diagnosis of *Angiostrongylus cantonensis* Infections in Humans Using Serologic and Molecular Methods

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## Abstract

Laboratory diagnosis of angiostrongyliasis relies on serological techniques, since definitive diagnosis is insensitive. Modern antibody detection methods focus on antibodies to the 29 and 31 kDa proteins of the parasite. Antigen detection may ultimately prove to be more reliable than antibody detection but no method has been adopted for clinical diagnostic use. Diagnosis using PCR amplification of DNA sequences specific to *Angiostrongylus cantonensis* have been developed but have not yet been validated for clinical use. Diagnostic tests have not been developed commercially and in the United States tests developed experimentally by non-commercial laboratories have to be approved by the Food and Drug Administration before they can be sold to other laboratories for diagnostic purposes.

## Keywords

Angiostrongyliasis, Diagnosis, Eosinophilic meningitis, Molecular methods, Serological methods

## Introduction

*Angiostrongylus cantonensis* is a leading cause of eosinophilic meningoencephalitis. Angiostrongyliasis is often suspected when eosinophilic pleocytosis is observed in cases of acute meningitis, particularly if accompanying risk factors, such as residence or travel to an area known to be endemic for the parasite, are also present.<sup>1,2</sup> A more conclusive diagnosis can be established by direct observation of the parasite in cerebrospinal fluid (CSF) or by using laboratory tests specific for *Angiostrongylus*-specific antibodies or parasite DNA.

Direct observation of *A. cantonensis* in the CSF is not common.<sup>3,4</sup> Third stage larvae may be present in the brain and CSF one to three weeks after infection. Third stage larvae are slender, measuring 460-520  $\mu\text{m}$  long by 22-27  $\mu\text{m}$  wide, with a rhabditoid esophagus and a tail that constricts before tapering to a blunt end.<sup>5</sup> Juvenile worms, which may be seen in brain biopsy and also in the vitreous of the eye, can be morphologically distinguished from other nematodes, such as *Gnathostoma spinigerum*, that might be found in the CSF based on size. The parasite continues to grow in the human host, and may rarely approach the size of adult worms found in rats.<sup>6</sup>

## Antibody and Antigen Detection

Because *A. cantonensis* is not reliably observed in the CSF of patients with angiostrongyliasis, laboratory diagnosis has historically relied on immunodiagnostic methods to detect parasite specific antibodies. Immunodiagnostic methods for angiostrongyliasis were employed in the 1960s soon after *A. cantonensis* was determined to be the probable etiologic agent of eosinophilic meningitis in Asia and the Pacific. An early diagnostic test was an intradermal test based on a skin reaction

to adult *A. cantonensis* extracts.<sup>3</sup> Results were indicative of angiostrongyliasis if the reaction to adult *A. cantonensis* extracts was three times greater than the reactions to a phosphate buffer control and to *Gnathostoma spinigerum*, *Paragonimus westermani*, *Dirofilaria immitis*, and *Toxocara canis*. Positive reactions were frequently elicited in asymptomatic individuals or patients with other parasitic infections.<sup>7</sup>

Various laboratory methods that focused on detection of *Angiostrongylus*-specific antibodies were developed in the 1970s, including indirect haemagglutination, complement fixation, indirect fluorescent antibody staining of frozen worm sections, lipid extracts of adult *A. cantonensis*, and latex agglutinations tests.<sup>8</sup> Enzyme linked immunosorbent assay (ELISA) methods were developed in the late 1970s and used crude antigen extracts prepared from young adult *A. cantonensis*.<sup>9</sup> Yen and Chen described ELISAs using partially purified *A. cantonensis* extracts prepared from either juvenile or adult *A. cantonensis*.<sup>10</sup> To reduce non-specific reactions, immunoabsorption was used to remove cross-reacting antigens of *Toxocara canis*, *Ascaris lumbricoides*, and *Clonorchis sinensis*. Both the juvenile worm and the adult worm preparations performed similarly in these experiments; neither preparation performed with significantly greater sensitivity or specificity than the other.<sup>10</sup>

Specificity continued to plague antibody detection methods so scientists sought to identify individual protein antigens that might be more specific than total worm extracts. Immunoblot studies demonstrated that serum antibodies from most patients with angiostrongyliasis specifically recognized the 29 kDa and 31 kDa proteins that are present in adult worm preparations.<sup>11-15</sup> In one report, the 31 kDa protein was more specific than the 29 kDa protein, with minimal cross reactivity from antibodies generated by other commonly encountered tissue-invading helminths.<sup>13</sup> Specific antibodies are present in serum and CSF so either specimen can be used for immunodiagnosis; however, detection of serum antibodies to the 31 kDa protein was reportedly more sensitive than CSF antibodies in one study.<sup>10</sup>

Purification of the 31 kDa protein by electroelution from SDS-polyacrylamide gels resulted in a highly sensitive and specific ELISA.<sup>16</sup> The purified 31 kDa protein was used to develop a dot blot assay for use in regional hospitals in Thailand.<sup>17</sup> An inter-laboratory evaluation of the dot blot assay proved that the method was easy to perform and results were reliable and reproducible across nine regional hospital laboratories.<sup>17</sup> The purified 31 kDa antigen has subsequently been incorporated into a multiplex assay for diagnosis of angiostrongyliasis with success.<sup>18</sup>

Although most studies have focused on the 29 kDa and 31 kDa antigens, detection of antibodies to other *A. cantonensis* proteins may also prove to be equally sensitive. Monoclonal antibodies have been used to purify a 204 kDa protein from subadults (stage 5) that was 91% sensitive and 98% specific in patients with eosinophilic meningitis.<sup>19</sup>

Several studies have evaluated specific immunoglobulin subclass responses, either to *A. cantonensis* crude somatic extracts or, in one study, to the 29 kDa protein specifically.<sup>20,21</sup> Specific IgG1 was the most sensitive class of immunoglobulin for immunodiagnosis of angiostrongyliasis using an *A. cantonensis* somatic antigen preparation.<sup>20</sup> In another study, detection of IgG4 specific for the 29 kDa *A. cantonensis* antigen was the most reliable subclass to measure, with a sensitivity of 75% and a specificity of 95%. Detection of *A. cantonensis* IgA and IgM were not useful.<sup>10,20</sup>

One important limitation of antibody detection for diagnosis is that serum antibody production follows acute symptom onset, sometimes significantly. In an outbreak in Jamaica in 2000, only 8% of acute phase serum specimens (collected 5-18 days after symptom onset) were positive, whereas 83% of the convalescent phase sera, (collected 31-45 days after symptom onset) were positive.<sup>22</sup>

Antigen detection in serum or CSF may ultimately prove to be more reliable than antibody detection for diagnosis of angiostrongyliasis. Several antigen detection methods have been reported in the literature, but none has been adopted for clinical diagnostic use. Monoclonal antibodies generated against adult *A. cantonensis* were used in an assay to detect *A. cantonensis* antigens in serum; this method was highly specific, but only 50% sensitive.<sup>23</sup> Another method was developed that detected a 204 kDa antigen that was present in both CSF and serum; detection in CSF was reported to be more sensitive than in serum.<sup>24</sup>

Immunodiagnostic tests for the detection of antibodies or antigens of *A. cantonensis* are not commercially available. Detection of antibodies to the 31 kDa protein is currently the assay of choice for immunodiagnosis of angiostrongyliasis but testing is available only at Mahidol University in Thailand at present. Proteomics approaches to purify the 31 kDa protein to generate recombinant forms of the protein are underway.<sup>25</sup> Availability of a recombinant form of the 31 kDa or other diagnostic proteins will make immunodiagnosis more widely available. Serologic testing for angiostrongyliasis is also available at the Khon Kaen University in Thailand, using assays based on detection of antibodies to the 29 kDa protein of *A. cantonensis*.

## Molecular Detection

Recently a conventional nucleic acid amplification test (NAAT) that amplified a 1,134 bp fragment from the *A. cantonensis* 18S rRNA gene and a real-time PCR assay (TaqMan) targeting the internal transcribed spacer-1 (ITS-1) were developed for detection of *A. cantonensis* in invertebrate hosts.<sup>26-28</sup> The TaqMan assay has also been used to support the diagnosis of

angiostrongyliasis in eosinophilic meningitis cases by detecting *A. cantonensis* DNA in CSF.<sup>29,30</sup> NAAT assays are not fully validated for clinical use at this time; therefore serological testing is still recommended to support NAAT results. NAAT testing for angiostrongyliasis is available at the Centers for Disease Control and Prevention (<http://www.dpd.cdc.gov/DPDx/HTML/Contactus.htm>).

## Regulatory Requirements

A discussion of diagnostic testing without some mention of the regulatory framework in the United States would be incomplete. Neglected diseases such as angiostrongyliasis do not attract sufficient resources from commercial test developers because costs are not rewarded with justifiable sales, so laboratories must develop their own tests. Performing laboratory developed tests (LDT) can be billed to clients, but the tests cannot be sold to other laboratories for diagnostic purposes (ie, as a kit) without U.S. Food and Drug Administration (FDA) approval.<sup>31</sup> Historically, regulation of LDTs has been within the Clinical Laboratory Improvement Amendments (CLIA) framework. Laboratories performing diagnostic testing must be certified by the Centers for Medicare and Medicaid Services (CMS) or a recognized organization such as a state health department or the College of American Pathologists (CAP), to be in compliance with CLIA. As such, developing laboratories must conduct formal verification to establish performance characteristics before results can be used for decisions regarding patient care. These performance characteristics include accuracy, precision, sensitivity, and specificity. Once analytical characteristics are determined, the clinical sensitivity and specificity must be verified in actual specimens. There is, however, no clear distinction between CMS and FDA jurisdiction. In fact, the FDA has asserted its authority over LDTs, categorizing them as medical devices in draft guidance documents as early as 1998.<sup>31</sup> The present FDA policy of exercising enforcement discretion by not regulating these tests as medical devices is uncertain, and may not be clarified until final guidance documents are published.

## Conflict of Interest

None of the authors identifies any conflict of interest.

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