

## Specificity of immunoblotting analyses in eosinophilic meningitis

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*Angiostrongylus cantonensis* and *Gnathostoma spinigerum* are the two most common causative parasites of eosinophilic meningitis (EOM). Serological tests are helpful tools for confirming the identity of the pathogen. Recent reports determined the specificity of such tests by using normal healthy controls. There have been limited studies done to rule out the cross-reactivity between these two causative parasites of EOM. This study aims to assess the specificity of the serological test in EOM by using each condition as a control for the other. Thirty-three patients with a diagnosis of EOM were enrolled. Sera from 22 patients with a positive 29-kDa antigenic diagnostic band of *A. cantonensis* were tested for the 21 and 24-kDa antigenic bands of *G. spinigerum*. Similarly, sera of 11 gnathostomiasis patients were tested for the 29-kDa diagnostic band for *A. cantonensis*. Only one patient in the angiostrongyliasis group had a positive result for the 21 and 24-kDa antigenic bands of *G. spinigerum*, while no gnathostomiasis patients showed a positive result for the 29-kDa antigenic band of *A. cantonensis*. The specificity of the 21 and 24-kDa antigenic bands for gnathostomiasis and the 29-kDa antigenic band for *A. cantonensis* was 95.5% and 100%, respectively. The antigenic bands for the diagnosis of gnathostomiasis and angiostrongyliasis in EOM were highly specific.

Key words: specificity - eosinophilic meningitis - immunoblotting - *Angiostrongylus cantonensis* - *Gnathostoma spinigerum*

Eosinophilic meningitis (EOM) is a condition clinically diagnosed by evidence of more than 10% of the total white blood cell count being eosinophils in cerebrospinal fluid (CSF) (Kuberski 2006, Wilson & Weller 2006, Sawanyawisuth & Sawanyawisuth 2008). It is an emerging condition and most commonly found in Southeast Asia, particularly in the Northeastern part of Thailand. Recently, it has been reported worldwide (Wang et al. 2008).

*Angiostrongylus cantonensis* and *Gnathostoma spinigerum* are the two main causative organisms of EOM (Punyagupta et al. 1990, Solomon et al. 2006). Even though both angiostrongyliasis and gnathostomiasis can be differentiated by clinical manifestations, the identity of the causative agent might be unclear or obscure in the diagnosis in some patients (Ramirez-Avila et al. 2009) and could lead to improper clinical diagnosis and treatment.

Several serological tests have been shown to be diagnostic tests for angiostrongyliasis and gnathostomiasis. Most studies of human EOM had normal healthy subjects or various helminthically infected subjects as controls (Nopparatana et al. 1991, Tapchaisri et al. 1991, Eamsobhana et al. 2001, Maleewong et al. 2001, Anantaphruti et al. 2005, Laummaunwai et al. 2007, Intapan et al. 2010). There are limited studies ruling out cross-reactivity between the two causative parasites of EOM.

Immunoblotting is a diagnostic method for both human angiostrongyliasis and gnathostomiasis. The detection of circulating antibodies against specific antigenic bands is the principle of the test. The widely used antigenic bands for angiostrongyliasis and gnathostomiasis are 29 and 21 or 24-kDa, respectively (Maleewong et al. 2001, Intapan et al. 2010). This study aims to show the specificity of diagnostic bands by immunoblotting for angiostrongyliasis and gnathostomiasis in eosinophilic meningitis. Patients with EOM caused by *A. cantonensis* will serve as controls for the diagnostic test of *G. spinigerum* and vice versa.

### PATIENTS, MATERIALS AND METHODS

The inclusion criteria for the study were patients diagnosed with EOM who had results of serological tests for both *A. cantonensis* and *G. spinigerum*. EOM was diagnosed if more than 10% of CSF eosinophils of the total CSF white blood cell count and if CSF staining for organisms, cryptococcal antigen testing and cultures was negative (Sawanyawisuth & Sawanyawisuth 2008).

Cases were defined as angiostrongyliasis if (i) there was a history of eating raw freshwater snails, freshwater prawns, crabs, frogs, monitor lizards or contaminated vegetables within the past three months and (ii) there were positive serum antibodies to the 29-kDa antigenic polypeptide of *A. cantonensis* (Maleewong et al. 2001).

A diagnosis of gnathostomiasis was made if the patients had a history of exposure to *G. spinigerum* larvae and had positive serum antibodies to the 21-kDa or 24-kDa antigenic polypeptide of *G. spinigerum* (Intapan et al. 2010). A history of eating or contact with raw freshwater fish, poultry, snakes or frogs was considered a risk factor for gnathostomiasis. In addition, a history of mi-

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gratory swelling, radicular pain or suggestive radiologic brain imaging such as track-like intracerebral haemorrhaging was supporting evidence for gnathostomiasis.

Sera of all patients were tested for specific antibodies against the 21 and/or 24-kDa antigenic bands of *G. spinigerum* and the 29-kDa band of *A. cantonensis* by immunoblotting. The methods of the technique are described below and are composed of two steps: (i) the creation of antigenic bands by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and (ii) the detection of antibodies against the specific polypeptide band by immunoblot analysis.

**SDS-PAGE** - Crude somatic antigen of young adult female (YAF) *A. cantonensis* or advanced third stage (aL3) *G. spinigerum* used in the SDS-PAGE technique (Laemmli 1970) was prepared according to the method as described by Maleewong et al. (2001). Briefly, YAF *A. cantonensis* from rats or aL3 *G. spinigerum* from mice were homogenised in distilled water containing a mixture of proteinase inhibitors followed by ultrasonic disintegration. The supernatant of the antigen was collected after centrifugation and then the protein was concentrated and lyophilised. The crude somatic antigen of *A. cantonensis* or *G. spinigerum* was then separated by molecular mass in 10-18% gradient separating or 12% SDS-PAGE gels, respectively. The molecular weight of the polypeptide bands of antigen were estimated by comparing their mobility to the standard molecular weight markers.

**Immunoblot analysis** - This technique is composed of two steps: electrophoresis transfer blot and enzyme immunoassay (EIA). The components of the polypeptide bands of each antigen on SDS-PAGE gels were blot electrotransferred onto nitrocellulose membranes according to the method of Towbin et al. (1979), with some modification. The transfer blot sheet was cut into 0.4 x 5.5 cm strips for detection of specific antibodies in each serum sample. The EIA process on nitrocellulose membranes was determined by the method previously described (Maleewong et al. 2001, Laummaunwai et al. 2007). Briefly, non-specific binding sites on the antigen strip were blocked by soaking in a solution of 1% skim milk in phosphate buffer solution with Tween 20 (blocking buffer) for 30 min. After washing with blocking buffer, each strip was incubated with the optimal diluted solution of each serum sample for 2 h. The strips were washed with blocking buffer, probed with an optimal diluted solution of goat anti-human IgG labelled horseradish peroxidase conjugate (Zymed Laboratories Inc) for 2 h and then washed again with the blocking buffer. The colour of the reactive polypeptide bands was developed after reacting with 3,3'-diaminobenzidine tetrahydrochloride substrate solution for 5 min. The reaction was stopped by washing in DW and air drying. Positive pooled sera of angiostrongyliasis or gnathostomiasis were used as positive controls. Negative pooled sera of healthy persons were used as negative controls. The negative controls were persons who had no clinical diagnosis of EOM and who had negative results against the 21, 24 and 29-kDa antigenic bands.

To determine the specific antibody to the 29-kDa polypeptide antigen of *A. cantonensis*, a serum dilution at 1:100 and a conjugate dilution at 1:5,000 revealed the best resolution, while the specific antibody against the 21 and/or 24-kDa polypeptide antigen of *G. spinigerum* gave a high resolution band with a serum dilution of 1:200 and a conjugate dilution of 1:15,000.

**Data analysis** - The cross reaction of the 29-kDa antigenic polypeptide of *A. cantonensis* against the sera of gnathostomiasis patients was determined and *vice versa*. The specificity of each diagnostic band was calculated.

**Ethics** - The study protocol was reviewed and approved by the institutional review board and the Ethical Committee of Khon Kaen University.

## RESULTS

Thirty-three patients with a diagnosis of EOM were enrolled. Of those, 22 had positive results for the 29-kDa antigenic diagnostic band of *A. cantonensis*, whereas 11 had positive results for the 21 or 24-kDa antigenic band of *G. spinigerum*. Sera of 22 angiostrongyliasis patients were tested for the 21 and 24-kDa diagnostic bands of *G. spinigerum*. Similarly, sera of 11 gnathostomiasis patients were tested for the 29-kDa diagnostic band of *A. cantonensis*.

Only one patient in the angiostrongyliasis group had a positive result for the 21 or 24-kDa antigenic bands of *G. spinigerum* (Table I), while no gnathostomiasis patients showed a positive result for the 29-kDa antigenic band of *A. cantonensis* (Table II). The specificity of the 21, or 24 and 29-kDa antigenic bands for gnathostomiasis and angiostrongyliasis in EOM was 95.5% and 100%, respectively.

TABLE I

A 2 x 2 table of immunoblotting analysis of the 21 or 24-kDa antigenic band for *Gnathostoma spinigerum* in eosinophilic meningitis by using eosinophilic meningitis patients caused by *Angiostrongylus cantonensis* as controls

	Gnathostomiasis (n = 11)	Angiostrongyliasis (n = 22)
Positive 21 or 24-kDa	11	1
Negative 21 or 24-kDa	0	21

TABLE II

A 2 x 2 table of immunoblotting analysis of the 29-kDa antigenic band for *Angiostrongylus cantonensis* in eosinophilic meningitis by using eosinophilic meningitis patients caused by *Gnathostoma spinigerum* as controls

	Angiostrongyliasis (n = 22)	Gnathostomiasis (n = 11)
Positive 29-kDa	22	0
Negative 29-kDa	0	11

## DISCUSSION

EOM in Southeast Asia and worldwide is mainly caused by either *A. cantonensis* or *G. spinigerum* (Solomon et al. 2006). Most serological diagnostic studies for these two parasites had very high specificity, but used normal subjects as controls (Nopparatana et al. 1991, Tapchaisri et al. 1991, Eamsobhana et al. 2001, Maleewong et al. 2001, Anantaphruti et al. 2005, Laummaunwai et al. 2007, Intapan et al. 2010). We herein confirm that the 21 or 24-kDa antigenic diagnostic band for gnathostomiasis and the 29-kDa antigenic diagnostic band for angiostrongyliasis in EOM are highly specific.

Recently, both 21 and 24-kDa antigenic bands have been shown to be specific for neurognathostomiasis (Intapan et al. 2010). Only one patient with a positive 29-kDa antigenic band for *A. cantonensis* had a positive result for the 21 or 24-kDa antigenic band of *G. spinigerum*. Three possible explanations are as follows: (i) the patient had been infected by *G. spinigerum* or *A. cantonensis* in the past because IgG was used in immunoblot analysis, (ii) there might be cross reaction between *A. cantonensis* and *G. spinigerum* or (iii) the patient had a coinfection from eating raw freshwater shrimp which contained both *A. cantonensis* and *G. spinigerum* larvae.

There are other possible causes of eosinophilic meningitis, such as *Toxocara canis* or *Baylisascaris procyonis* (Solomon et al. 2006). However, both of these conditions are very rare. By clinical features, history of larvae exposure and geographic location, these two parasites can be differentiated from *A. cantonensis* and *G. spinigerum*. In addition, both parasites are rare causes of eosinophilic meningitis; they usually cause eosinophilic meningoencephalitis, which is more severe and presents with dramatically altered mental status. Most patients with EOM have a normal mental status (Solomon et al. 2006).

Eosinophilic meningoencephalitis caused by *T. canis* has been reported in two children who had history of contact with puppy faeces (Moreira-Silva et al. 2004), while *B. procyonis* was also reported in children who had contact with raccoon faecal matter (Chun et al. 2009, Page et al. 2009). In a literature review, there are a very limited number of cases in which these two parasites caused only eosinophilic meningitis. Very rarely, *Paragonimus westernmani* may be a cause of eosinophilic meningitis. However, the central nervous system complication of *P. westernmani* is usually accompanied by cavitary lung lesions and brain calcification or a "soap bubble" appearance (Solomon et al. 2006).

Even though the specificity of the 29-kDa antigenic band for angiostrongyliasis is very high, the sensitivity is only 55.6%. A negative result of the test is not excluded in the diagnosis of angiostrongyliasis in clinically suspected patients. Another limitation of this study was the small number of subjects tested for all antigenic bands. The specificity of the tests might be lower if more subjects were enrolled. Further cohort study may be needed to confirm the results.

In conclusion, clinicians can be confident of the specificity of the antigenic bands for the diagnosis of *A. cantonensis* and *G. spinigerum* in eosinophilic meningitis.

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