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# A dot-blot ELISA comparable to immunoblot for the specific diagnosis of human parastrongyliasis

**P. Eamsobhana*, A. Yoolek, P. Punthuprapasa and S. Suvouttho**

Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

## Abstract

A dot-blot enzyme-linked immunosorbent assay (dot-blot ELISA) using an electroeluted 31-kDa glycoprotein from adult worms of *Parastrongylus cantonensis* as the specific antigen was evaluated for the immunological diagnosis of patients infected with *P. cantonensis*. The sensitivity and specificity for the detection of serum antibody to *P. cantonensis* in dot-blot ELISA were both 100%, as determined with serum samples of ten *P. cantonensis*-infected patients, 60 patients with other related parasitic infections, and 20 uninfected controls. The test was as sensitive and specific as the immunoblot test which revealed a reactive band of 31 kDa. Both the dot-blot ELISA and immunoblot detected all sera from ten *P. cantonensis*-infected individuals, but not with those of other heterologous parasitoses (gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis and malaria) or sera from healthy controls. The dot-blot ELISA is much simpler to perform than the immunoblot technique, and the test can be applied under field conditions where sophisticated facilities are lacking.

## Introduction

Eosinophilic meningitis or eosinophilic meningoencephalitis caused by *Parastrongylus* (= *Angiostrongylus*) *cantonensis*, continues to pose a public health problem in the world (Cross, 1987; Alicata, 1991; Kliks & Palumbo, 1992; Durette-Desset *et al.*, 1993; Wariyapola *et al.*, 1998; Chotmongkol & Sawanyawisuth, 1999; Prociv *et al.*, 2000; Tsai *et al.*, 2001; Slom *et al.*, 2002). The difficulty in finding the parasite in the cerebrospinal fluid (CSF) renders it virtually impossible to confirm the presence of *P. cantonensis* in many patients suspected to be infected with this neurotrophic metastrongylid nematode (Punyagypta, 1979). In recent years, various immunological assays have been developed for detecting human parastrongyliasis (= angiostrongyliasis). The enzyme-linked immunosorbent assay (ELISA) is used most commonly and has yielded promising results (Chen, 1986; Ko, 1987; Kliks *et al.*, 1988).

The immunoblot is more precise than the ELISA for detection of infectious diseases because positive reactions are determined not only by colour reaction but also by the antigen profile. Generally, only a limited number of specific bands are required for accurate diagnosis of positive reactions. Immunoblot analysis to detect antigen–antibody complexes is used as a confirmatory test for *Parastrongylus* infection because of its accuracy (Eamsobhana *et al.*, 1997, 1998; Slom *et al.*, 2002). In our laboratory, the immunoblot procedure has been used for more than five years to validate results of conventional micro-ELISA using crude somatic extracts from adult worms of *P. cantonensis* as antigen. The immunoblot test is more specific than the traditional colorimetric ELISA using crude antigens because the band pattern on the immunoblot can be interpreted as positive (reactive with a specific 31-kDa band) or negative (non-reactive). However, the major drawback of immunoblot analysis is that the procedure is relatively cumbersome and requires an appropriately equipped laboratory. The equipment required and its high cost limit widespread application in rural settings.

The dot-immunobinding assay, a modification of the ELISA using a nitrocellulose membrane as a test matrix, is rapid, specific, sensitive and easy to perform and is commonly used in simple qualitative research applications. A dot ELISA-type immunodiagnostic test,

*Fax: +66 (0) 2411 2084
E-mail: sipes@mucc.mahidol.ac.th

detecting either antibodies or antigens, has been used successfully for immunodiagnosis of a variety of parasitic infections (Brooks *et al.*, 1985; Gandhi, 1986; Tellez-Giron *et al.*, 1987; Zheng *et al.*, 1990; Oprandy & Long, 1990; Allan *et al.*, 1993; Rabello *et al.*, 1993). In the present study, a dot-blot assay is described using a purified 31-kDa antigen that is comparable to the immunoblot analysis and has specificity and sensitivity similar to the immunoblot test.

## Materials and methods

### *Preparation of crude soluble extract*

The Thailand strain of *P. cantonensis* was used in the present study. Procedures for larval collection, infection of rats, adult worm collection and crude antigenic preparation were similar to those described by Eamsobhana *et al.* (2001). In brief, adult worms obtained from the pulmonary arteries of infected rats were washed and homogenized in a small volume of normal saline with a glass tissue grinder. The suspension was sonicated and left overnight at 4°C to allow elution of antigen. Soluble antigen was obtained as the supernatant after centrifugation at 5000 rpm for 15 min. The protein content of the extract was determined using the protein assay kit II (Bio-Rad Laboratories, USA).

### *Preparation of electroeluted antigen*

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and isolation of a 31-kDa specific antigen from SDS-polyacrylamide gels by electroelution were carried out as described by Eamsobhana *et al.* (2001). Briefly, a 1.5-mm single trough of a 12% separating gel was loaded with about 2.0 mg of adult worm extract of *P. cantonensis*. Separate slots in the same gel were used to electrophorese the high and low molecular weight standards. When the run was completed, strips with the molecular standards were cut and rapidly stained with Coomassie brilliant blue R 250 to determine the region where the 31-kDa antigen of interest would be according to the approximate molecular weight. The region containing the 31-kDa targeted antigen was cut into strips and the gel pieces were electroeluted in an electroeluter (Model 422, Bio-Rad) using a clear membrane cap at a molecular weight cut off of 12,000-15,000 Daltons. Elution was done at 10 mA/glass tube constant current for 3 h. Electroeluted protein was desalted and concentrated by ultrafiltration using Centricon YM-10 (Millipore Corporation, USA). The protein content was estimated using Protein Assay Kit II (Bio-Rad). The electroeluted fraction containing the 31-kDa specific protein of *P. cantonensis* was used in the dot-blot ELISA for the detection of specific antibody to *P. cantonensis*.

### *Serum samples*

Serum samples were obtained from five patients with parasitologically proven parastrongyliasis (three with cerebral parastrongyliasis from whom *P. cantonensis* larvae were recovered from the CSF; two with ocular parastrongyliasis from whom immature *P. cantonensis* worms were recovered from their eye chambers) and five patients with presumptive parastrongyliasis. The latter group was diagnosed as parastrongyliasis based on clinical symptoms and history of exposure to infection, as well as having high antibody titres as detected by ELISA.

Antigen cross-reactivity studies were carried out on sera from ten patients each with gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis, and malaria. All these cases were positive by parasitological and/or serological tests for a specific parasite or its products. Two patients with gnathostomiasis and all patients with filariasis, paragonimiasis and malaria were diagnosed parasitologically. Ten cases of toxocariasis were serologically positive on ELISA using excretory–secretory antigens of the second-stage larvae of *Toxocara canis*, while patients with cysticercosis were diagnosed pathologically as subcutaneous cysticerci. The normal control group of sera were obtained from 20 healthy adults who were negative for any parasitic infection at the time of blood collection. All serum samples were kept at − 20°C until use.

### *Dot-blot ELISA procedure*

An electroeluted fraction containing the 31-kDa specific protein of *P. cantonensis* was used in the dot-blot ELISA for detecting specific antibodies to *P. cantonensis*. The optimal antigen concentration per dot and the appropriate serum dilution were pre-determined by chequerboard titration.

A nitrocellulose membrane (0.45 $\mu$m pore size; Bio-Rad Laboratories, Richmond, California) was cut into pieces (1.5 × 1.5 cm), soaked for 5 min in phosphate-buffered saline (PBS), pH 7.4 and air dried on filter paper. Antigen adsorption was carried out by spotting 2 $\mu$l of 31-kDa antigen fraction (0.2 $\mu$g protein per dot) of *P. cantonensis*, diluted in PBS, in the centre of each piece of membrane and allowed to dry for 1 h. Membranes were then immersed in blocking solution (5% skimmed milk in PBS) with gentle shaking for 1 h. After washing three times, by shaking for 5 min during each wash in PBS-Tween 20, the membrane was incubated for 1 h in each serum to be tested (diluted 1 in 200 with 1% bovine serum albumin (BSA) in PBS) and again washed as described above. The washed membranes were immersed in the enzyme conjugate, peroxidase rabbit anti-human immunoglobulins (Dakopatt, Denmark) diluted 1:1,000 with 1% BSA in PBS for 1 h. These membranes were washed as previously described and transferred to a substrate solution containing 30 mg of 4-chloro-1-naphthol (Pierce Chemical Company, USA) in 10 ml of absolute methanol mixed with 100 $\mu$l of 30% $H_2O_2$ in 100 ml of PBS, pH 7.4. After the appearance of the colour, the membranes were washed with distilled water for several times. The development of well-defined blue dots on the nitrocellulose membrane was considered as positive. No colour-dots were considered as negative.

The same serum samples were tested in triplicate to confirm reproducibility of the results.

### *Electrophoresis and immunoblotting*

Crude antigenic extract of *P. cantonensis* was separated on a 12% reducing SDS–polyacrylamide slab gel. After

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electrophoresis, the resolved polypeptide bands were electrophoretically transferred from the gel to a nitrocellulose membrane for immunoblotting, as described by Towbin *et al.* (1979). The non-specific binding sites on the membrane were blocked by soaking in a solution of 5% skimmed milk in phosphate-buffered saline (PBS), pH 7.4 for 1 h. The membrane was then incubated with test serum, which was diluted 1:200 in 1% bovine serum albumin (BSA) in PBS, pH 7.4, overnight at 4°C. After washing thoroughly the membrane was then reacted with horseradish peroxidase-conjugated rabbit anti-human immunoglobulins (Dakopatt, Denmark) at a dilution of 1:1000 in PBS, pH 7.4 for 1 h at room temperature followed by washing. The bound antigen-antibody complexes were visualized by adding the chromogenic substrate solution containing 30 mg of 4-chloro-1-naphthol (Pierce Chemical Company, USA) in 10 ml of absolute methanol mixed with 100 $\mu$l of 30% $H_2O_2$ in 100 ml of PBS, pH 7.4. The blots were rinsed in distilled water, air-dried and photographed.

### *Statistical analysis*

Sensitivity and specificity of the tests were calculated using the formula given by Galen (1980).

## Results

All serum samples were tested by the dot-blot ELISA and immunoblot procedures. Both the number of positive reactions in dot-blot ELISA and the intensity (colour) of the positive reactions agreed well with those of the immunblot test which revealed a reactive band of 31-kDa. All ten serum samples from patients with parastrongyliasis gave deep blue colour dots (positive test). None of the other 60 sera with gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis and malaria and the 20 normal control sera showed a positive blue dot. Both the sensitivity and specificity of the tests were 100%. The results of the dot-blot ELISA using purified 31kDa antigen against sera with parastrongyliasis, other related parasitic infections and normal controls are shown in fig. 1.

Immunoblot analysis of the crude soluble extract from adult worms of *P. cantonensis* against sera with parastrongyliasis and other related parasitic infections, and uninfected controls, are shown in fig. 2. Sera from *P. cantonensis*-infected patients reacted strongly with isolated antigens to show a prominent band of 31 kDa (lanes A-J). This 31-kDa antigen was not recognized by sera from patients with gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis and malaria, and sera from normal healthy individuals (lanes K–R).

## Discussion

Detection of *P. cantonensis* larvae in CSF to confirm infection may not be successful because of low larval intensity. In the present study, a rapid, simple and cheap immunological test of high sensitivity and specificity for detecting antibody against *P. cantonensis* 31-kDa antigen is described. The dot-blot ELISA employed in this study was based on the detection of an antigen-antibody complex on nitrocellulose membrane and the electroeluted, purified antigen was used to eliminate nonspecific immunological reactions. All sera from patients with parastrongyliasis contained the antibody identified by this 31-kDa antigen, and this was readily detected with a relatively simple colorimetric dot immunobinding assay. In contrast, none of the 60 sera from patients with other parasitic infections and 20 normal individuals yielded positive test results under these conditions. The dot-blot ELISA using purified specific antigen has a sensitivity and specificity of 100%.

The present study demonstrated that the dot-blot ELISA test using electroeluted, purified antigen was specific and had good sensitivity, comparable to the standard immunoblot assay. Both the dot-blot assay and immunoblot analysis confirmed the presence of antibodies to *Parastrongylus* worms in five serum samples of infected individuals that were negative by parasitological diagnosis. The dot-blot ELISA and immunoblot consistently detected all parasitologically confirmed and clinically diagnosed *P. cantonensis*-infected patients. The dot immunobinding technique described herein has sensitivity and specificity similar to that of the immunoblot procedure, which revealed a specific band of 31 kDa. The improved specificity of dot-blot ELISA was obtained through antigen purification by SDS–PAGE and electroelution which eliminated most cross-reacting antigens. Furthermore, the treatment of antigens with 2-mercaptoethanol and heating denature the antigens and expose

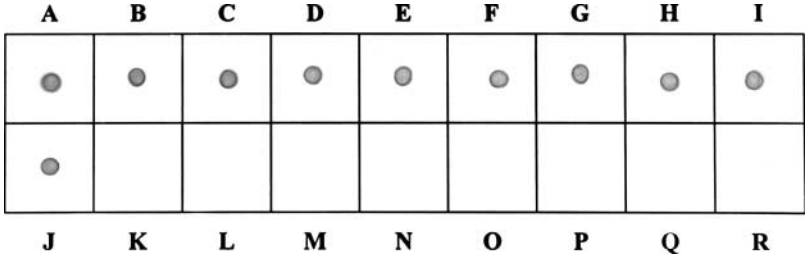


Fig. 1. Reactions of dot-blot-ELISA using an electroeluted, purified 31-kDa antigen of *Parastrongylus cantonensis* on nitrocellulose membrane for the detection of specific antibodies to *P. cantonensis* from sera of patients with parastrongyliasis (A–J), sera from patients with gnathostomiasis (K), toxocariasis (L), filariasis (M), paragonimiasis (N), cysticercosis (O), and malaria (P), and normal control sera (Q, R), using electroeluted, purified 31-kDa antigen of *P. cantonensis*. A–J, deep coloured dots showing positive reaction; K–R, no colour was considered as negative.

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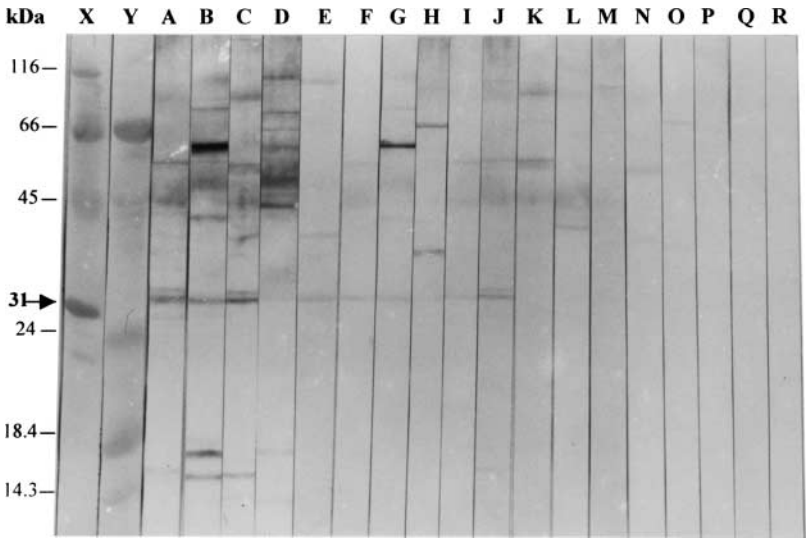


Fig. 2. Immunoblot analysis showing reactivity of serum samples from patients with parastrongyliasis (A–J), gnathostomiasis (K), toxocariasis (L), filariasis (M), paragonimiasis (N), cysticercosis (O) and malaria (P), and normal control sera (Q, R) against crude worm extract of *Parastrongylus cantonensis* antigen. X and Y are high and low molecular weight standards (Sigma). The arrow indicates a *P. cantonensis*-specific 31-kDa band.

the epitopes that might not be accessible to antibodies in their natural conformations on the antigens. This process exposes the antigen determinants in the dot ELISA similar to those in immunoblot; therefore, the dot-blot ELISA resembles the immunoblot.

An ideal diagnostic test for use in a small-scale epidemiological study should be simple and easy to perform in addition to being both sensitive and specific. The use of purified 31-kDa antigen with dot-blot ELISA will contribute considerably to the performance and dependability of this test under normal field conditions. For the dot-blot ELISA described herein, no sophisticated electrical equipment is required as a positive reaction is observed by eye with reliability. The assay can be completed in 4 h, and more than 100 samples can be tested each time. Although the preparation of purified antigen requires additional time, the dot-blot ELISA test utilizes minute amounts of antigen. Of the 12 mg protein of the crude extract of *P. cantonensis* initially applied for electrophoresing, after electroelution, about 0.49 mg protein (4.08% yield) of the purified antigen was obtained (Eamsobhana *et al.*, 2001). This amount of antigen (0.49 mg) is sufficient to test more than 2500 samples. Production of this specific 31-kDa protein by recombinant DNA techniques undoubtedly will improve the procedure of antigen preparation, thereby making it more cost-effective.

The present study shows that the dot-blot ELISA test using purified antigen is a promising tool for sensitive, specific and rapid diagnosis of infection with *P. cantonensis*. Further studies will be concerned with the evaluation of its applicability for epidemiological studies.

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