

BLINDED MULTI-LABORATORY EVALUATION OF AN IN-HOUSE DOT-BLOT ELISA KIT FOR DIAGNOSIS OF HUMAN PARASTRONGYLIASIS

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Abstract. An in-house membrane dot-blot enzyme-linked immunosorbent assay kit, for the detection of specific antibody to a 31 kDa protein of *Parastrongylus cantonensis* was evaluated in a blinded multi-laboratory study. The kit was provided to technologists, who were trained in its use, from nine regional laboratories of Thailand. With an identical set of 20 coded serum samples and reference positive and negative controls, the kit was found to have a diagnostic sensitivity of 100% and a diagnostic specificity of 100% in all the laboratories. There was no obvious variation in quality among five lots of the antigen-coated nitrocellulose strips evaluated. The shelf life of the kit was ≥ 6 months when the test components were stored at 4°C and 18 months at -20°C. The enzyme-linked immunoassay dot technique is easy to perform and does not require sophisticated electrical equipment; the result is available within 3 hours. If appropriate technical training is included, the application of this dot-blot ELISA kit in clinical laboratories throughout Thailand should be possible.

INTRODUCTION

Parastrongylus (= *Angiostrongylus*) *cantonensis* is a major causative agent of eosinophilic meningitis and eosinophilic meningoencephalitis in humans throughout much of the Asia-Pacific region (Cross, 1987; Alicata, 1991; Kliks and Palumbo, 1992; Wariyapola *et al*, 1998; Chotmongkol and Sawanyawisuth, 1999). A definitive diagnosis of human parastrongyliasis (angiostrongyliasis), based on the detection of parasites in the patients' cerebrospinal fluid or eyes, is rarely possible (Punyagypta, 1979). The need to improve immunodiagnostic testing for parastrongyliasis is therefore pressing, particularly in areas where human infections are common. Many diagnostic techniques are available, but the major problems of laboratory diagnosis that are faced by many developing countries, *eg* the lack of specific reagents and

equipment, make the techniques impracticable. Until recently the reagents for most parasitic diseases, including parastrongyliasis, have not been commercially available. Therefore diagnostic services have been restricted to a very small number of well-established, well-equipped laboratories. This is one of the reasons for the under-reporting of cases and the poor state of disease surveillance. The development of a simple diagnostic kit is important; the ideal kit should be suitable for use in the field and in smaller hospitals and clinics.

The use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting has been shown to be a reliable way of detecting antibody to *Parastrongylus*-specific 31 kDa protein in sera of patients (Eamsobhana *et al*, 1997; 1998). The clinical value of the 31 kDa glycoprotein of *P. cantonensis* became clear when the plate enzyme-linked immunosorbent assay (ELISA) and membrane dot-blot ELISA were developed, using an electroeluted, purified 31 kDa glycoprotein as the specific antigen, in order

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to confirm the presence of *P. cantonensis* infection in humans (Eamsobhana *et al*, 2001; 2002). Its usefulness led to the development of a simplified test kit using an enzyme-linked immunoassay dot technique. To further assess the performance of our test kit, a blinded multi-laboratory study for the detection of antibody to *Parastrongylus*-specific 31 kDa protein was conducted.

During 7-8 August 2000, the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University conducted a workshop entitled "Application of immunological and molecular biological techniques in the diagnosis of parasitic diseases" in co-operation with the Bureau of Laboratory Quality Standards, Department of Medical Sciences, Ministry of Public Health, Thailand. The workshop aimed to instruct laboratory personnel from 21 regional hospitals and 3 regional medical sciences centers in the recently-developed advanced techniques for the diagnosis of medically important parasitic diseases in Thailand. Instruction included an introduction to the use of commercially available test kits for malaria and filariasis. Upon completion of the course, the dot-blot ELISA technique for the diagnosis of parastrongyliasis was produced as a test kit, which was provided, along with a sample set of coded sera, to 9 selected participants, all of whom were from areas in which *P. cantonensis* infection is endemic. This paper describes the evaluation of the test kit.

MATERIALS AND METHODS

Participating laboratories

Nine diagnostic laboratories, all situated in areas endemic for *P. cantonensis* infection, were selected for this evaluation: eight regional hospital laboratories (Khon Kaen, Phitsanulok, Saraburi, Buri Rum, Surin, Ubon Ratchathani, Prachin Buri and Loei Provinces) and one clinical pathology laboratory (Regional Medical Sciences Center, Khon Kaen). All the technologists from these collaborating labora-

tories had participated in the workshop "Application of immunological and molecular biological techniques in the diagnosis of parasitic diseases" (see above) and had been trained in the use of the dot-blot ELISA procedure.

Clinical and control specimens

A set of 20 sera selected for specific anti-*P. cantonensis* antibody testing contained: the sera of three patients with parasitologically-confirmed parastrongyliasis; one serum each of gnathostomiasis, filariasis, strongyloidiasis, trichinosis, paragonimiasis, taeniasis, opisthorchiasis, amebiasis, and malaria; and the sera of eight normal non-parasitized individuals. All samples were assigned a random number. Pooled sera from confirmed parastrongyliasis patients and healthy individuals negative for any parasitic infection at the time of blood collection, were used as reference positive and negative controls. These sera had been subjected to prior testing of anti-*Parastrongylus* 31 kDa specific antibody by the standard immunoblot assay: the results matched the parasitological data. One sample set, along with a test kit, was given to each participant for testing in their own laboratories; the sample sets and test kits were packed in an ice-box.

Dot-blot ELISA kit

Test kits were prepared at the Department of Parasitology, Faculty of Medicine Siriraj Hospital. The assay used a nitrocellulose-based dot-blot technique for the detection of antibody to *P. cantonensis* specific 31 kDa protein (Eamsobhana *et al*, 2002). The antigen used was an electroeluted fraction containing the 31 kDa specific protein of *P. cantonensis* prepared by the method described previously (Eamsobhana *et al*, 2001).

The kit consisted of strips of nitrocellulose membrane (0.45 µm pores; Bio-Rad Laboratories, USA); each strip (8.0 x 1.0 cm) was covered by a plastic mask with 5 circular windows, on to the center of each of which the antigen was deposited as a discrete dot (0.2 µg protein/dot). These nitrocellulose strips were blocked with 5% skimmed milk in phosphate

buffered saline (PBS), packed in plastic bags, sealed, and kept at -20°C . Each test kit was accompanied by the necessary reagents and buffers; detailed instructions for performing the test were provided. The contents of the kit are shown in Table 1.

Dot-blot ELISA procedure

The enzyme-linked immunoassay dot technique followed the step-by-step procedure described by Eamsobhana *et al* (2002).

The nitrocellulose antigen-coated strip, which was in a plastic pack, was removed from the -20°C freezer or 4°C refrigerator. The test strip was pre-wetted with PBS (pH 7.4), dried, and placed on a hydrophobic (paraffin) film, to which was applied $20\ \mu\text{l}$ of each serum sample (1:100 in blocking buffer) to different windows on the strip. The strip was placed in a moist Petri dish and incubated for one hour at 37°C . After washing the strip three times in washing buffer for 15 minutes, $20\ \mu\text{l}$ of the 1,000-fold diluted peroxidase conjugated anti-human immunoglobulins (Dakopatt, Denmark) was introduced into each window on the strip and again incubated for one hour at 37°C in a moist Petri dish. The strip was washed and transferred into a substrate solution containing 15 mg of 4-chloro-1-naphthol (Bio-Rad) in 5 ml of absolute methanol mixed with $15\ \mu\text{l}$ of

$30\% \text{H}_2\text{O}_2$ in 25 ml of PBS (pH 7.4) and incubated in the dark for 15 minutes. The strip was washed with distilled water and dried.

Kit diagnostic criteria

The participating technologists were instructed to read the test strip by following the kit instructions, using the intensities of the reactive and non-reactive controls as guides. A positive reaction appeared as a well-defined bluish-purple dot in the window on the test strip. If no dot was seen, the reaction was interpreted as negative.

Kit stability and reproducibility

Five lots of the antigen-coated strips were prepared exactly as described above. The shelf life of the kit was determined by periodically testing the antigen strips, kit reagents, and control sera monthly for the first year, and again at 14, 16 and 18 months. Monitored antigen strips and reference serum samples were kept at 4°C and -20°C , while monitor kit reagents were stored at 4°C throughout the study.

Data analysis

The sensitivity and specificity of the test were determined by standard methods (Galen, 1980).

Table 1
Contents of the dot-blot ELISA kit for parastrongyliasis diagnosis.

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| 1. Antigen-coated strips: 5 antigen dots/strip; 10 strips. Strips transported at 4°C and stored at -20°C . |
| 2. Buffered diluent/washing buffer: PBS (pH 7.4) 1 packet; NaCl 8.56 g, KH_2HPO_4 0.23 g, Na_2HPO_4 1.18 g. To be dissolved in 1,000 ml distilled water. |
| 3. Blocking buffer: 5 g skimmed milk (1 packet). To be dissolved in 100 ml buffered diluent. Stored at 4°C . |
| 4. Conjugate: horseradish peroxidase conjugated rabbit anti-human immunoglobulins (Dakopatt, Denmark), 1 vial ($20\ \mu\text{l}$). Reagent transported and stored at 4°C . |
| 5. Chromogen: 4-chloro-1-naphthol (Bio-Rad, USA), 1 vial (75 mg). Transported and stored at 4°C . |
| 6. 30% Hydrogen peroxide, 1 vial (1 ml). Transported and stored at 4°C . |
| 7. Positive and negative serum controls, 1 vial each ($20\ \mu\text{l}$). Transported at 4°C and stored at -20°C . |
| 8. A set of 20 coded serum samples, 1 vial each ($20\ \mu\text{l}$). Transported at 4°C and stored at -20°C . |
| 9. Package insert with complete instructions. |

RESULTS

All the nine participating laboratories correctly reported positive anti-*Parastrongylus* 31 kDa antibody in three coded serum samples from patients with parastrongyliasis. Negative results were reported for the coded test samples of gnathostomiasis, strongyloidiasis, filariasis, trichinosis, paragonimiasis, opisthorchiasis, taeniasis, amebiasis, and malaria sera, and the 8 normal controls. Among the individual laboratories, there were no qualitative differences in sensitivity and specificity when the dot-blot ELISA kit was performed with the same set of serum samples. The sensitivity and specificity of the dot-blot ELISA were 100%, according to every laboratory.

When antigen-coated strips and positive reference serum samples stored at either 4°C or -20°C were periodically tested for 18 months, no significant change in levels of positive reactivity was noticed during the first six months of evaluation, under both storage conditions. Positivity dropped when antigen strips and sera were kept in excess of 6 months at 4°C. No variation in positive reactivity was noted for storage at -20°C during the 18-month period. Negative control sera remained non-reactive throughout the study. In addition, no obvious variation in the results was observed when sera were tested using five different lots of antigen-coated nitrocellulose sheets.

DISCUSSION

In rural Thailand, most patients with parastrongyliasis are admitted to peripheral hospitals, which lack sophisticated laboratory facilities. These patients must be managed without the benefit of a specific diagnosis, partly because of the lack of a simple, reliable diagnostic test that can be used in basic laboratories. These problems have resulted in efforts to develop sensitive, specific, inexpensive, practical, and rapid diagnostic tests which can be performed in small local laboratories, eliminating the need to send specimens to a reference facility, and thus speeding diagnosis.

Progress in developing new diagnostic techniques for parasitic diseases has been dramatic in the past decade. Although technological improvement has been achieved for applications to field conditions, few techniques have met the four basic requirements for use in developing countries: simplicity, rapidity, accuracy and economy.

Immunoblot assay has been previously designed to detect the anti-31 kDa specific antibody in the sera or CSF of patients with eosinophilic meningitis caused by *P. cantonensis* (Eamsobhana *et al*, 1997; 1998). Immunoblotting, long the reference method for the diagnosis of human parastrongyliasis, is technically demanding and is not practicable in less equipped laboratories. In Thailand, it is only performed in our own laboratory and a few other university hospital laboratories, where sophisticated electrical equipment is available. The development of a more rapid test, based on the detection of specific anti-*P. cantonensis* antibody, that is suitable for use in most clinical laboratories in Thailand is important; such a test deserves an objective evaluation of its qualities.

The dot-immunobinding assay is one of several variations of a diagnostic technique that is based on visualization of a specific reaction with target molecules, such as an antigen-antibody reaction on synthetic membranes. It is technically simple, does not depend on expensive equipment, and results can be obtained relatively rapidly. A dot-blot ELISA kit for *P. cantonensis* specific antibody using an electroeluted, purified 31 kDa antigen was developed and found to have a sensitivity and specificity of 100% for diagnosis of parastrongyliasis in our preliminary evaluation (unpublished data). Nevertheless, for an evaluation of diagnostic kits, it is best to conduct a multi-center study, and the results are interpreted blindly (Lina *et al*, 1996). To ascertain the performance of our dot-blot ELISA kit, we conducted a blinded multi-laboratory study: the results obtained by nine individual laboratories were identical. The kit was judged to have 100% sensitivity and 100% specificity for

the diagnosis of parastrongyliasis. Although diagnostic accuracy may be variable relative to experience with the test in routine daily use, in the present evaluation all the participating technologists were trained in the use of the kit prior to performing the test in their own laboratories. Not surprisingly, there was no difference in the quality of results among the laboratories. This suggests that the initial training in kit use was adequate.

In evaluating the qualities of diagnostic kits that depend on subjective visual reading, selection and composition of a panel of specimens used, however, affect the outcome (Vaughn *et al*, 1998). This is because visual perception, required for discriminating positive from negative specimens, varies between readers and, most importantly, is influenced by the titer of positive specimens. In this evaluation, the three proven positive sera from a sample set consisted of highly positive sera: they yielded intense color reactions in the dot test, resulting in a sharp contrast between these samples and the negative ones. Positive sera were always interpreted correctly. As the most critical element for membrane-based diagnostic kits is subjectivity caused by variation in visual perception among diagnosticians, with further inclusion of moderate-to-low positive visual guides to ensure the positive interpretation, the application of this dot-blot ELISA kit in peripheral hospital laboratories in Thailand should be possible.

Although some weaknesses remain, in the hands of trained personnel, this dot-blot ELISA kit for *P. cantonensis* antibodies provides a sensitive, specific, reproducible and practical semi-quantitative product that can be used in middle-level diagnostic laboratories that are in areas in which human parastrongyliasis is common.

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