A partially purified specific antigen of *Parastrongylus cantonensis* for immunodiagnosis of human parastrongyliasis

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Abstract

Three distinct protein peaks were obtained when the crude extract of *Parastrongylus cantonensis* adult worms was passed through a Sephacryl S-200 HR column. The antigenic components of the three isolated peak fractions were studied by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblot analysis using serum from a patient with parasitologically confirmed parastrongyliasis. The 31-kDa diagnostic antigen of *P. cantonensis* was demonstrated mainly in the first elution profile. The band of 31-kDa antigenic component reacted strongly with parastrongyliasis serum. A semi-purified fraction from the first elution peak was used in the indirect enzyme-linked immunosorbent assay (ELISA) for detecting specific antibody to the 31-kDa antigen of *P. cantonensis*. A total of 200 serum samples was used in the study. Of these, 25 sera were from patients with parastrongyliasis. Fifteen sera each were from patients with gnathostomiasis, toxocariasis, filariasis, paragonimiasis and cysticercosis. The control group consisted of 100 serum samples from normal healthy individuals. The sensitivity and specificity of the ELISA were 100% and 98.87%, respectively. This Sephacryl S-200 fraction containing an immunodominant antigen of 31 kDa, appears promising as a diagnostic reagent in the ELISA for human parastrongyliasis.

Key words: *Porastrongylus* (=*Angiostrongylus*) *cantonensis*; gel filtration chromatography; immunoblotting; semi-purified antigen; ELISA

Introduction

Parastrongylus (*syn. Angiostrongylus*) *cantonensis* is an important causative agent of eosinophilic meningoencephalitis in man in Southeast Asia and Pacific islands (Punyagupta, 1979; Cross, 1987; Koo *et al.*, 1988). The diagnosis of parastrongyliasis (=angiostrongyliasis) is generally presumptive since the parasite can only infrequently be recovered from the cerebrospinal fluid (CSF) of patients. Numerous techniques have been used for the immunodiagnosis of human parastrongyliasis (Tharavanij, 1979; Welch *et al.*, 1980; Ko, 1987; Kliks *et al.*, 1988). Of these methods, the enzyme-linked immunosorbent assay (ELISA), a simple immunological test, requires the detection of Parastrongylus antibodies. The test is dependent upon antigen quality, specificity and sensitivity. The antigenic reagents currently available for use in ELISA for detecting *P. cantonensis* infection in humans still lack the necessary specificity. This is probably due, in part, to the extensive antigenic overlap that exists not only between nematodes but also between nematodes and other parasites (Tharavanij, 1979; Ko, 1987; Chen, 1986; Dharmkrong-at *et al.*, 1986). There is therefore the need for a more specific antigen for the assay.

In the last few years significant progress has been made in the development and improvement of immunological techniques for laboratory diagnosis of *P. cantonensis* infection. Recently, the nature of the antigens related to the specific antibodies produced by the host has been explored. Akoa *et al.* (1992) demonstrated by immunoblots that the 29-kDa and 31-kDa proteins in adult female worm of *P. cantonensis* were specific antigens recognized by infected humans. In a similar study, Eamsobhana (1994) showed that a specific antigen with relative molecular weight of 31 kDa, found in the crude extracts from adult male and female worms and third-stage larva of *P. cantonensis*, was consistently identified by sera from patients infected with *P. cantonensis*. This immunodominant 31-kDa antigen was further demonstrated to be a glycoprotein and its carbohydrate moiety did not reveal any active epitope (Eamsobhana *et al.*, 1998).

Although the ELISA is widely used for immunodiagnosis of nematode diseases including

parastrongyliasis, there have been very few studies of ELISA using fractionated antigens and none using characterized specific antigens from *P. cantonensis*. In the present study we attempted to isolate the 31-kDa diagnostic antigen from the crude worm extract of adult *P. cantonensis* by gel filtration chromatography using Sephacryl S-200 HR column. The purity and specificity of the eluted fractions were then characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using serum from a patient with parasitologically confirmed parastrongyliasis. The isolated fraction containing the 31-kDa antigen was further used to determine the reactivity of the sera in the ELISA to specifically detect the corresponding (anti-31-kDa) antibody. The present results indicate that this semi-purified antigen of *P. cantonensis* can be used in a diagnostic ELISA for human infection, and that this antigen shows better specificity than that previously reported using crude parasite antigen (Eamsobhana *et al.*, 1999).

Materials and Methods

Preparation of crude antigens

The Thailand strain of *P. cantonensis* was used in the present study. Adult worms were obtained from the pulmonary arteries of albino rats that were infected for at least 6 weeks with *P. cantonensis* infective larvae collected from experimentally infected snails, *Biomphalaria glabrata*. Male and female worms were washed and then homogenized in a small volume of normal saline with a glass tissue grinder. The suspension was then sonicated and left overnight at 4°C to allow elution of antigens. Soluble antigens were obtained in the supernatant after centrifugation at 4000 rpm for 15 min. The protein content of the extracts was determined using a protein assay kit II (Bio-Rad Laboratories, Hercules, CA, USA).

Gel filtration chromatography

The crude antigens from adult *P. cantonensis* were separated by size exclusion gel filtration chromatography using Sephacryl S-200 HR (Pharmacia, Uppsala, Sweden) as chromatographic matrix on a 1.6 x 100 cm C16/100 column (Pharmacia). About 20 mg of antigenic sample (2 ml) were applied to the Sephacryl S-200 column equilibrated with PBS, pH 7.2. Fractions of 3 ml/tube were collected at a flow rate of 25 ml/h using a fraction collector (Pharmacia). The absorbance of each elute (3 ml) was measured at 280 nm. Fractions belonging to the same peak were pooled and concentrated by ultrafiltration through a PM10 membrane (Amicon Inc., Beverly, USA). Protein concentration was determined using a protein assay kit II (Bio-Rad). The partially purified antigens were further assayed by SDS-PAGE and immunoblotting for the detection of specific 31-kDa component.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Components of crude and partially purified antigens were separated on SDS-polyacrylamide slab gels using the discontinuous system of Laemmli (1970). A 4% stacking gel and 12% separating gel were used. Protein samples of about 10µg were boiled at 100°C for 5 min in Laemmli sample buffer before loading onto the gel. The separated protein bands were visualized by Coomassie brilliant blue staining. The molecular weight of separated proteins was estimated by comparing their electrophoretic mobilities with those of known standard molecular weight markers (Sigma Chemical Company, St. Louis, USA) in the same gel. For transblotting, the SDS-PAGE separated antigens from the gel were electroblotted onto a 0.45 µm nitrocellulose membrane (Bio-Rad) by the method of 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 reacted with horseradish peroxidase conjugated goat 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 of the chromogenic substrate solution containing 60 mg of 4-chloro-1-naphthol (Bio-Rad) in 20 ml of cold absolute methanol mixed with 60 µl of 30% H_2O_2 in 100 ml of PBS, pH 7.4. The blots were rinsed in distilled water, air-dried and photographed.

Enzyme-linked immunosorbent assay (ELISA)

A concentrated Sephacryl S-200 fraction containing the 31-kDa specific protein of *P. cantonensis* was used in ELISA for the detection of specific antibody to *P. cantonensis*.

The ELISA was performed according to the method of Voller *et al.* (1976) with some modifications. Briefly, wells of microtiter plate (Nunc, Denmark) were sensitised with 100 µl of a fractionated *P. cantonensis* antigen at a concentration of 2 µg/ml of protein in

carbonic buffer solution, pH 9.6. The wells were successively incubated for 2 h each with 100 µl of blocking solution (2% skimmed milk in PBS-Tween 20), serum samples diluted to 1:400 with PBS containing 1% bovine serum albumin and 0.05% Tween 20, and peroxidaseconjugated anti-human immunoglobulins (Dakopatt, Denmark) diluted to 1:4000 in PBS-Tween. Finally, the wells were incubated for 30 min with the substrate (o-phenylenediamine) solution. The enzymatic reaction was stopped with 50 µl of 2.5 N sulphuric acid and the optical density (OD) was measured at 492 nm with an ELISA reader (SLT Labinstrument, Australia).

The optimal concentration of the antigen and the optimal dilution for patient's serum and conjugate were pre-determined using a chequerboard titration. For each test, negative, positive and PBS-Tween controls were included.

A result was considered positive if the OD value exceeded the mean OD+6SD of the values obtained with the 100 negative sera.

Serum samples

Human sera were collected from five patients with parasitologically confirmed parastrongyliasis (three with cerebral parastrongyliasis from whom *P. cantonensis* larvae were recovered from the CSF; the other two had ocular parastrongyliasis from whom immature *P. cantonensis* worms were recovered from their eye chambers) and 20 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 titers as detected by ELISA.

Seventy-five heterologous sera were obtained from patients with other parasitic infections. Of these, 15 sera each were from patients with gnathostomiasis, toxocariasis, filariasis, paragonimiasis and cysticercosis. All these cases were positive by parasitologic and/or serologic tests for a specific parasite or its products. The normal control group of sera was obtained from 100 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.

Results

The chromatographic profile obtained showed the elute divided into three separated peaks, consisting of one broad low peak, and two sharp and narrow peaks with higher absorbances (Fig. 1). The protein content of the three isolated fractions was 3.9, 3.5 and 1.9 mg, respectively. About 46.5% of the applied protein was recovered.

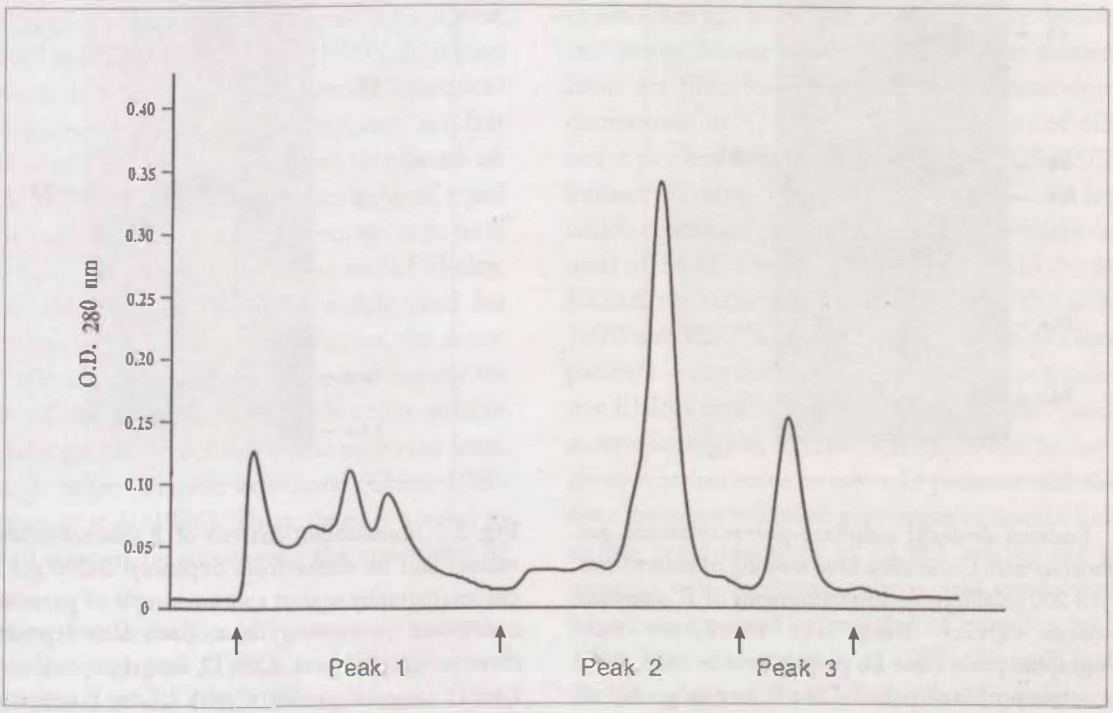

Fig. 1. Fractionation of *Parastrongylus cantonensis* adult worm extract by gel filtration chromatography on a Sephacryl S-200 HR column. The y-axis shows the optical density at 280 nm.

The electrophoretic pattern of the protein components of the three Sephacryl S-200 fractions as revealed by SDS-PAGE is shown in Fig. 2. SDS-PAGE and Coomassie blue staining did not reveal a clear single protein band with molecular weight of 31 kDa in any of the three protein profiles. The first elution profile contained about 9 visible protein bands with molecular weight ranging from 22 to 110 kDa (lane D), the second profile contained 7 protein bands with molecular weight from 12.5 to 64 kDa (lane E), while the third profile contained 4 protein bands with molecular weight from 18.5 to 22 kDa (lane F).

The antigenic components of the crude adult worm extracts and the eluted fractions were determined by SDS-PAGE and immunoblot analysis against the serum from one of the five parasitologically confirmed human parastrongyliasis. As shown in Fig. 3, the serum contained high concentration of specific antibody to the 31-kDa protein (lane C). With the same serum, the 31-kDa diagnostic protein of *P. cantonensis* was demonstrated mainly in the first Sephacryl S-200 profile (lane D).

The isolated fraction from the first elution peak, which contained mainly the specific protein of 31 kDa, was used in the indirect ELISA for determining the specific antibody to *P. cantonensis* in serum samples from healthy individuals and from patients with parastrongyliasis and other parasitic infections. The mean OD±SD of the normal group was 0.073±0.027. The mean OD+6SD of the healthy group sera (i.e. OD > 0.235) was taken as the positive cut-off value.

All 25 serum samples from parastrongyliasis cases (100%) were positive in the ELISA and 73 of 75 sera (97.33%) from patients with other parasitic infections were negative (Fig. 4). Cross-reactions were found with serum samples from gnathostomiasis (2/15). Normal parasite-free individuals were all negative. The sensitivity and specificity of the ELISA were 100% and 98.87%, respectively.

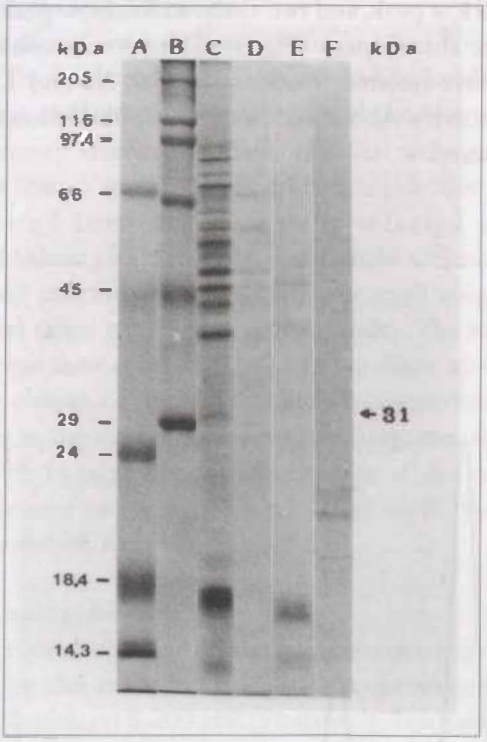

Fig. 2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis with Coomassie blue staining of elutes from Sephacryl S-200 gel filtration chromatography of *P. cantonensis* adult worm extract. Each lane represents one chromatographic peak. Lane D, protein profile of peak 1; Lane E, protein profile of peak 2: Lane F, protein profile of peak 3. Lane C shows the protein profile of crude adult worm extract of *P. cantonensis*. Lanes A and B are low and high molecular weight markers.

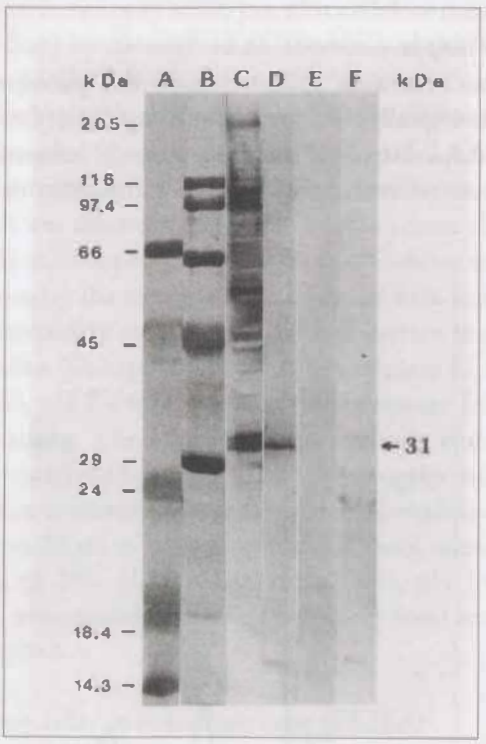

Fig. 3. Immunoblot analysis of *P. cantonensis* adult worm extract and its elutes from Sephacryl S-200 gel filtration chromatography against a serum sample of parasitologically confirmed parastrongyliasis. Each lane represents one chromatographic peak. Lane D, antigenic profile of peak 1; Lane E, antigenic profile of peak 2; Lane F, antigenic profile of peak 3. Lane C shows the antigenic profile of crude adult worm extract of *P. cantonensis*. Lanes A and B are low and high molecular weight markers.

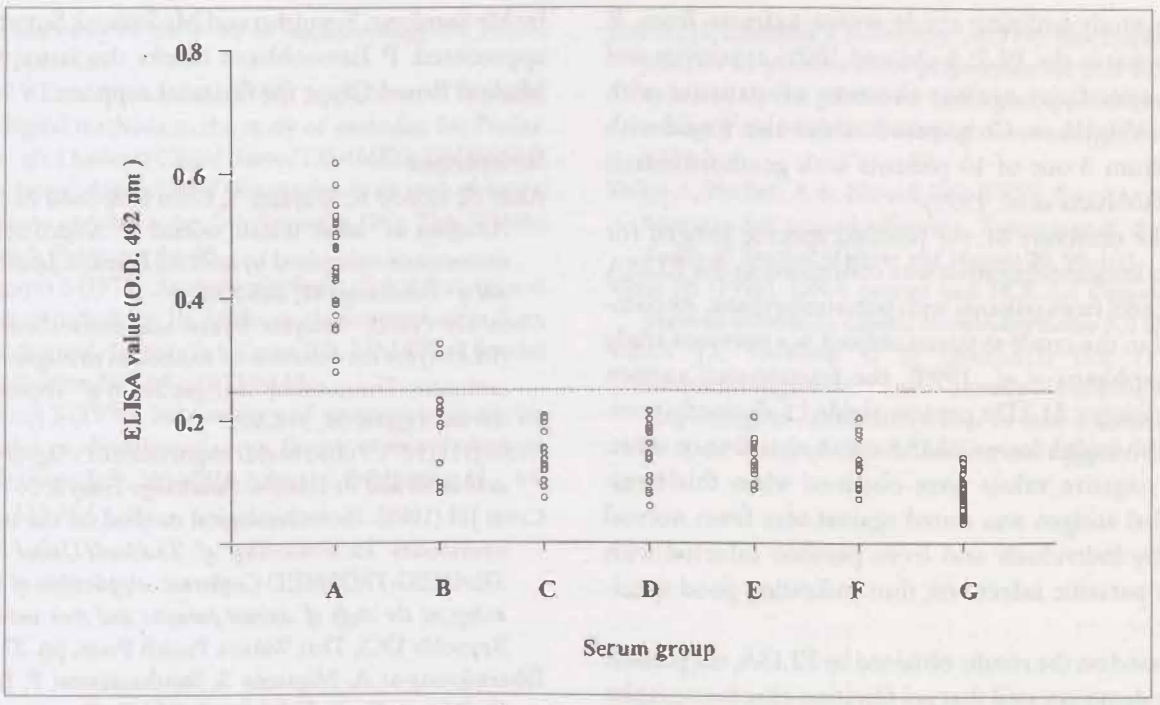

Fig. 4. Distribution of optical density values in ELISA for detection of a specific 31-kDa serum antibody against *P. cantonensis* in 25 patients with parastrongyliasis (A), 15 patients each with gnathostomiasis (B), toxocariasis (C), filariasis (D), paragonimiasis (E), and cysticercosis (F), and in 100 normal healthy individuals (G). The dotted line shows the cut-off value.

Discussion

Although molecular techniques are capable of differentiating very small quantities of material for the specific identification of many human parasites (Cross, 1988; Murrell & Rhoads, 1988; Weiss, 1995), there have been no reports of their use in the specific identification of *P. cantonensis* in humans. A cheap, easy and fast alternative is to use an immunological test based on the ELISA. The ELISA has been shown to be of equal value to or superior to other immunodiagnostic tests for a wide range of parasites and is the test of choice. At present, although the ELISA is widely used for immunodiagnosis of *P. cantonensis* infection, the sensitivity and specificity of the assay depend mostly on the quality of the antigens used. With crude soluble antigens, false positive reactions occur with sera from patients with other parasitic infections (Chen, 1986; Dharmkrong-at *et al.*, 1986). Thus, there is a need to use purified antigen for improving the specificity of the assay.

Recently a specific antigen of *P. cantonensis* with relative molecular weight of 31 kDa was identified and evaluated (Akao *et al.*, 1992; Eamsobhana, 1994; Eamsobhana *et al.*, 1997). The 31-kDa component has been shown to be a carbohydrate containing protein (Eamsobhana *et al.*, 1998). In the present study, the fractionation and purification of the 31-kDa glycoprotein from crude worm extracts involved Sephacryl S-200 HR column chromatography. Fractionation of crude adult worm antigens showed three distinct protein peaks detected at 280 nm. The first elution peak from gel filtration contained the immunodominant component of 31 kDa and trace amount of other reactive protein antigens as revealed by SDS-PAGE and immunoblotting. When this Sephacryl S-200 fraction, which contained primarily the specific protein component of 31 kDa was used as an antigen in the indirect ELISA, the sensitivity and specificity of the assay were 100% and 98.87%, respectively. Although two sera from patients with presumptive gnathostomiasis gave positive ELISA results with this semi-purified *P. cantonensis* antigenic reagent, the reaction might not be due to the cross-reaction since another 13 patients with *Gnathostoma spinigerum* infection gave negative results. Relatively strong cross-reactions in ELISA among the sera of patients with parastrongyliasis and gnathostomiasis have been reported by a number of investigators (Chen, 1986; Dharmkrong-at *et al.*, 1986). Since *P. cantonensis* and *G. spinigerum* are clinically-related parasites, a positive ELISA using crude antigenic preparations with the serum of a patient suspected of harbouring *G. spinigerum* needs careful interpretation. Likewise, a pre-

vious study utilizing crude worm extracts from *P. cantonensis* in the ELISA showed 100% sensitivity and 94% specificity against the sera of patients with parastrongyliasis. Cross-reaction was also found with sera from 3 out of 10 patients with gnathostomiasis (Eamsobhana *et al.*, 1999).

The reliability of the purified specific antigen for use in immunodiagnosis was confirmed in the ELISA using sera from patients with parastrongyliasis. As compared to the crude antigens utilized in a previous study (Eamsobhana *et al.*, 1999), the fractionated antigen containing a 31-kDa protein yielded a distinctly positive although a lower ELISA mean absorbance value. Low negative values were obtained when this semi-purified antigen was tested against sera from normal healthy individuals and from persons infected with other parasitic infections, thus indicating good specificity.

Based on the results obtained by ELISA, the present study demonstrated that gel filtration chromatography is a useful method for the isolation of the specific *P. cantonensis* component. The limitation for this technique is the difficulty of collecting sufficient quantities of specific antigen for large-scale immunodiagnostic purposes. Only 3.9 mg partially purified protein was eventually recovered from the initial 20 mg protein of the crude worm extract, representing a 19.5% yield.

This is the first step in purification of an immunodominant specific antigen from crude antigens for immunodiagnosis of human parastrongyliasis. As the 31-kDa specific antigen is present only in small quantity from the crude worm antigens, further purification is needed to obtain a single component of 31 kDa. Attempts should also be made to produce monoclonal antibody against the 31-kDa antigen. The purification of a suitable parasite-specific antigen and the availability of a monospecific antibody would open the possibility of producing a specific diagnostic reagent in sufficient quantity, probably through recombinant DNA technology.

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