DETECTION OF ANTIBODIES TO *PARASTRONGYLUS CANTONENSIS* IN HUMAN SERA BY GELATIN PARTICLE INDIRECT AGGLUTINATION TEST

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**Abstract:** A newly developed agglutination test using gelatin particles as an antigen carrier (GPAT) was compared with a conventional enzyme-linked immunosorbent assay (ELISA) for the detection of *Parastrongylus cantonensis* antibodies in sera from patients. A total of 70 sera was used in the study. Of these, 10 each were from patients with parastrongyliasis, gnathostomiasis, paragonimiasis, cysticercosis, toxocariasis, filariasis and malaria. The control group consisted of 50 serum samples from normal healthy individuals. The mean reciprocal titer of the parastrongyliasis patients group was significantly higher than that of the normal group as well as those of other parasitic infections. The sensitivity and specificity of the GPAT were 100% and 92.4%, respectively. The results of GPAT in detecting *P. cantonensis* antibodies appeared to be closely correlated with those obtained with ELISA. The GPAT, however, is more easy, rapid and cheap; it may also be a test of choice for routine immunodiagnosis of human parastrongyliasis.

**Key words:** Immunodiagnosis, *Parastrongylus (= Angiostrongylus) cantonensis*, gelatin particle indirect agglutination test (GPAT), ELISA

**INTRODUCTION**

Human eosinophilic meningitis or meningoencephalitis caused by *Parastrongylus (= Angiostrongylus) cantonensis* is endemic throughout Asia and the Pacific Islands (Cross, 1987; Kliks and Palumbo, 1992). The most reliable diagnosis of this parasitic disease is based on the presence of either larvae or juvenile worms in the cerebrospinal fluid (CSF) from the patients. Such diagnosis nevertheless is rare since worms are seldom found in the limited volume of CSF taken for analysis.

A variety of immunological tests based on detecting serum antibodies against *P. cantonensis* has been used to support the diagnosis. These include intradermal test, indirect hemagglutination test, immunodiffusion, immunoelectrophoresis, complement fixation test, ELISA and immunoblot test (Tharavanij, 1979; Ko, 1987; Eamsobhana et al., 1997). The enzymatic test system has become more widely used because of its greater sensitivity. The test, nevertheless, requires specific materials, specialized equipment and expensive reagents. Recently, a newly developed agglutination test using gelatin particle as an antigen carrier has been shown to be a sensitive and specific method for the diagnosis of human strongyloidiasis (Sato and Ryumun, 1990), schistosomiasis (Yang et al., 1994; Kobayashi, 1995), Chagas' disease (Yamashita et al., 1994) and opisthorchiasis (Watthanakulpanich et al., 1998). GPAT is technically simple and can be performed rapidly without specialized apparatus or facilities. These make it convenient to use in the diagnosis of many diseases both in the laboratories as well as in the field.

In this study, we attempted to evaluate whether GPAT could be used to detect serum antibodies of parastrongyliasis (= angiostrongyliasis) patients. The results were compared with those of ELISA.

**MATERIALS AND METHODS**

**Antigen preparation**

Adult worms of *P. cantonensis* were obtained from the pulmonary vessels of infected Wistar albino rats as...
previously described (Eamsobhana et al., 1997). The worms were homogenized in a small volume of normal saline with a glass tissue grinder. The suspension was sonicated and extracted overnight at 4°C in a refrigerator. Soluble antigens were obtained as supernatant after centrifugation at 4,000 rpm for 15 min. The protein content of the extract was determined using a protein assay kit II (Bio-Rad Labs, USA).

Sera

Serum samples were obtained from five patients with parasitologically confirmed parastrongyliasis (3 with cerebral parastrongyliasis; 2 with ocular parastrongyliasis) 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 titers as detected by ELISA.

Sixty heterologous sera were collected from patients suffering from other parasitic infections. Of these, 10 sera each were from patients with gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis and malaria. All these cases were positive by parasitologic and/or serologic tests for a specific parasite or its products. The normal control group of sera were obtained from 50 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.

Gelatin particle indirect agglutination test (GPAT)

The GPAT was performed as previously described (Watthanakulpanich, 1998). Briefly, the pre-determined optimal concentration of P. cantonensis antigens (50 μg/ml) was conjugated to the artificial gelatin particles (Fujirebio Inc., Tokyo, Japan) treated with 5 μg/ml tannic acid solution. After conjugation of antigens, the gelatin particles were washed 3 times and finally made into a 1% suspension in phosphate buffered saline (PBS), pH 7.0 containing inactivated normal rabbit serum. These coated gelatin particles were then ready for use.

For estimation of agglutination titer, one drop containing 25 μl of the antigen-coated particles suspension was mixed in the U-bottomed micro-wells with an equal volume of test serum in 2-fold serial dilutions. The particles were allowed to settle for at least 3 hr at room temperature and the agglutination patterns in the plates were read according to Campbell et al. (1974); particles spread out uniformly covering the bottom of the well indicated a positive result. The antibody titer was determined as the highest serum dilution giving a positive agglutination pattern.

Enzyme-linked immunosorbent assay (ELISA)

To evaluate the results of GPAT, the ELISA was also applied for assessment of serum antibodies to P. cantonensis.

The ELISA was performed according to the method described by Voller et al. (1976) with some modifications. Briefly, wells of microtiter plate (Nunc, Denmark) were sensitized with 100 μl of P. cantonensis antigens at a concentration of 5 μg/ml of protein in carbonic buffer solution, pH 9.6. The wells were successively incubated for 2 hr each with 100 μl of blocking solution (2% skim milk in PBS-Tween), serum samples diluted to 1:100 with PBS containing 1% bovine serum albumin and 0.05% Tween 20, and peroxidase-conjugated anti-human immunoglobulins (Dakopatt, Denmark) diluted to 1:1,000 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 antigens and the optimal dilution for patient's serum and conjugate were pre-determined using a chequerboard titration. For each test, a negative, a positive and a PBS-Tween controls were included.

A result was considered positive if the OD value exceeded the mean OD + 3SD of the values obtained with the 50 negative sera.

Statistical analysis

Sensitivity and specificity of the tests were determined using the method of Galen (1980). Association between GPAT and ELISA was evaluated using linear correlation and regression after the titers of GPAT were transformed into logarithm (log2).

RESULTS

The distribution of GPAT titers in 70 patients and 50 uninfected controls is shown in Figure 1. All 10 patient sera with parastrongyliasis showed positive agglutination response at serum titer of 1:32 or more (log2 reciprocal titer ≥ 5), whereas negative results were demonstrated at the lowest serum titer of 1:16 (log2 reciprocal titer ≤ 4) in all the normal individuals. The
log₂ reciprocal titers in the parastrongyliasis patients ranged from 5 to 8, with the majority from 6 to 7.

The mean antibody titer (X̄ ± SD) of normal healthy individuals was 3.15 ± 0.58. The mean antibody titer of parastrongyliasis patients group was significantly higher than that of the normal group as well as those of other parasitic infections (P < 0.01). A cut-off titer for the positive antibody response was then established at X̄ + 3SD of the healthy group which was at log₂ reciprocal titer of 4.89. The GPAT was positive for all the parastrongyliasis patients but negative for the normal controls.

Of the 60 serum samples from other groups of parasitic infections, 51 (85%) were negative, while 9 (15%) were cross-reactive at the cut-off titer. The cross-reactive serum samples were from patients with gnathostomiasis (5/10), toxocariasis (2/10), filariasis (1/10), and paragonimiasis (1/10). None of the cysticercosis and malaria patients showed cross-reaction. The sensitivity and specificity of the GPAT were 100% and 92.4%, respectively.

The ELISA was carried out using different dilutions of sera from healthy individuals and from patients with parastrongyliasis. The maximum difference in OD values was observed at 1:100 dilution which was used to evaluate all serum samples. The mean OD value (X̄ ± SD) of the normal group was 0.253 ± 0.107. The mean plus three standard deviation OD value of the healthy group sera was then taken as the cut-off value, OD > 0.574 indicated positive results.

As shown in Figure 2, all the serum samples from parastrongyliasis cases (10/10) were positive in the ELISA and 48 of 60 sera from patients with other parasitic infections (80%) were negative. Cross-reactions were found with serum samples from gnathostomiasis (6/10), toxocariasis (3/10), filariasis (1/10), and paragonimiasis (2/10). Normal parasite-free individuals were all negative. The sensitivity and specificity of the ELISA were 100% and 90.2%, respectively.

Figure 3 represents the correlation of GPAT titers (log₂) and ELISA values performed on 10 sera from parastrongyliasis patients.
the correlation coefficient was 0.968.

DISCUSSION

The definitive diagnosis of parastrongyliasis is made when Parastrongylus worms are found in the CSF of patients. Proven cases of human infection showing worms are however rare (Punyagupta, 1979) and the ELISA utilizing either crude or partially purified Parasstrongylus antigens is currently used as a reliable method for immunodiagnosis of the infection (Welch et al., 1980; Chen, 1986; Yen and Chen, 1991). Although ELISA is sensitive enough for detecting serum antibodies to P. cantonensis, it is expensive, laborious and limiting with respect to the length of time to get results.

The recently developed inert gelatin particles are actively being employed as an antigen carrier for various diagnostic kits, and GPAT has already been successfully applied by a number of investigators for immunodiagnosis of various parasitic infections (Sato and Ryumon, 1990; Yamashita et al., 1994; Yang et al., 1994; Kobayashi et al., 1995; Watanakulpanich et al., 1998). In the present study, we confirmed the findings that GPAT could also be used for immunodiagnosis of human parastrongyliasis. The sensitivity of the present GPAT did not differ from that of the ELISA when the overall rates of positive reactions among patients' sera diagnosed to be parastrongyliasis were compared; in sera of 10 patients with parastrongyliasis, positive antibody response was demonstrated in all of them by the GPAT and ELISA. When such tests were evaluated for specificity by testing sera from healthy individuals presumed to be normal, the result did not show any false-positive reactions in each test. A significant correlation was observed between GPAT and ELISA.

Sera from patients with other parasitic infections were also examined by the GPAT and ELISA to determine the specificity of the tests. Strong positive responses were observed in a few patients with gnathostomiasis. Weak cross-reactive positive reactions also occurred in sera from a few patients with toxocariasis, filariasis and paragonimiasis. The responses, however, were lower in the GPAT. The sera from patients with cysticercosis and malaria showed no cross-reactivity in both tests. The positive responses in gnathostomiasis, toxocariasis, filariasis and paragonimiasis patients, however, seem to correlate with the antigen preparation used rather than the assay itself. By using a more defined antigen, the cross-reaction can be expected to decrease (Ko, 1987).

The present study indicated that the GPAT can be a reliable immunological test for human parastrongyliasis. The indirect agglutination test is technically simple to perform, requires no specialized skill, equipment and facilities, and can be completed within three hours. The advantages of the gelatin particles are their stability and resistance to mechanical agitation. The particles are colored and therefore convenient for reading the setting pattern. Moreover, the particles can be lyophilized for long term storage after sensitization with antigen. Therefore, the GPAT can be performed by the one-step reaction of the preserved antigen-particles with a test serum, thus applicable both in less equipped laboratories as well as in a field survey. Nevertheless, because of the relatively strong cross-reaction with other clinically related parasites, Gnahtostoma spinigerum, the use of more specific antigenic preparation in the assay will be needed. Experiment on purification of the P. cantonensis specific antigen for future use is underway.

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