

Indirect haemagglutination test using monoclonal antibody-affinity purified antigens for diagnosis of human paragonimiasis due to *Paragonimus heterotremus*

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Summary

An indirect haemagglutination test (IHA) using antigens purified by monoclonal antibody-affinity chromatography was developed for the diagnosis of human paragonimiasis caused by *Paragonimus heterotremus*. Sera from patients with paragonimiasis ($n = 30$) were evaluated, along with sera from other parasitic infections ($n = 92$), pulmonary tuberculosis ($n = 18$) and healthy controls ($n = 30$). The sensitivity, specificity as well as positive and negative predictive value of the IHA, calculated at the prevalence of disease at 17.6%, were all 100%.

keywords paragonimiasis, indirect haemagglutination test, diagnosis, affinity purified antigens, *Paragonimus heterotremus*

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Introduction

Paragonimus heterotremus is one of several species of lung flukes which infect humans (Miyazaki & Harinasuta 1966; Miyazaki & Fontan 1970; Vanijanonta *et al.* 1981; WHO 1995). Laboratory diagnosis of human infection is made primarily by detection of worm eggs in the sputa and/or faeces. However, the result can be negative in early as well as extrapulmonary infections. Serodiagnostic tests, which demonstrate antibodies against *P. heterotremus*, play a supplementary role to the parasitological method. Antibodies against this lung fluke have been detected in human serum by several serological methods such as indirect haemagglutination test (IHA) and enzyme-linked immunosorbent assay (ELISA) using crude extract of *P. heterotremus* as the antigen (Pariyanonda *et al.* 1990; Maleewong *et al.* 1990). By immunoblot analysis, a specific component with relative molecular mass of 31.5 kD was identified for serodiagnosis of human paragonimiasis caused by *P. heterotremus* (Maleewong *et al.* 1991; 1992). However, the result of Indrawati *et al.* (1991) and Dekumyoy *et al.* (1995) show marked differences in specific antigens. Recently, monoclonal

antibody (MAb) against the 31.5 and 22 kD components of the excretory-secretory (ES) antigens has been produced and used in affinity chromatography to purify antigens (Maleewong *et al.* 1997). In the present study, the MAb-affinity chromatography purified antigens containing a 31.5 kD antigenic component was prepared and used in IHA for serodiagnosis of human paragonimiasis caused by *P. heterotremus*.

Materials and methods

Antigen preparations

The ES antigens of adult *P. heterotremus* were prepared as previously described (Maleewong *et al.* 1997). Briefly, adult worms were placed in tissue culture medium, RPMI-1640 containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.1 mM *N*-tosyl-L-phenylalanine-chloromethylketone (TPCK) and incubated at 37 °C under 5% CO₂ atmosphere for 12 h. The medium was replaced at 2-hourly intervals; the spent medium was pooled and concentrated by ultrafiltration using Amicon YM 3 membrane filter (Grace & Co., Dancers,

MA) and dialysed against distilled water containing 0.1 mM PMSF and 0.1 mM TPCK. The solution was clarified by centrifugation at 10,000 g for 30 min. The supernatant fluid containing crude ES antigen was passed through an affinity chromatography column containing Sepharose 4B coupled with the specific MAb 10F2 (Maleewong *et al.* 1997). Bound material was then eluted and used for the IHA below. The eluted materials consisted of 31.5 and 22kD protein as revealed by SDS-PAGE followed by Coomassie brilliant blue staining.

Sera

Thirty paragonimiasis sera were obtained from villagers in the endemic area of Thailand whose sputa contained *P. heterotremus* eggs. All had a history of eating raw crabs from mountain streams in the area of endemic paragonimiasis 3 to 6 months before blood collection. The clinical symptoms consisted of bronchitis with gelatinous, tenacious, rust-brown, pneumonic-like golden flakes and blood-streaked sputum. Sera from patients with opisthorchiasis, opisthorchiasis with other parasitic infections, gnathostomiasis, angiostrongyliasis, trichinosis, cysticercosis, schistosomiasis, capillariasis and fascioliasis were obtained from either parasitologically confirmed cases or subjects whose stools contained either helminth eggs or larvae upon examination by formalin-ether concentration technique. Tuberculosis sera were obtained from patients whose clinical findings were compatible with pulmonary tuberculosis and whose sputa were positive for acid-fast tubercle bacilli.

Negative control sera were taken from apparently healthy volunteers living in a non-endemic area. Their stool examinations at the time of blood collections were negative for any parasites or their derivatives (such as ova or larva).

Pooled positive reference serum was prepared by combining equal volumes of each paragonimiasis serum. Pooled negative reference serum was prepared similarly from negative sera.

IHA procedures

An indirect hemagglutination test was performed as described by Morakote *et al.* (1984). Briefly, glutaraldehyde-treated human group O erythrocytes sensitized with affinity-purified ES antigens were used. Human group O blood was collected in Alsever's solution and stored at 4 °C for at least 2 days. After extensive washing with 0.15 M phosphate-buffered saline (PBS), pH 7.2, a 10 % (v/v) cell suspension was made. In 4 parts of the cell suspension, 1 part of 2.5 % glutaraldehyde in PBS, pH 7.2, was added drop by drop with constant stirring. The reaction was allowed to take place at room temperature for 2 h. Then treated cells were washed 3

times with the buffer solution and finally resuspended to 2.5 % (v/v) concentration.

Tanning and sensitization followed procedures described elsewhere (CDC 1976). Tanning took place in an ice bath, the sensitization with antigen at 37 °C. The final cell suspension was 0.75 % in PBS, pH 7.2 containing 0.5 % bovine serum albumin (BSA) (Sigma, St Louis, Mo, USA) and 0.1 % gelatin. The preparation remained stable at 4 °C for at least 6 months.

Tests were carried out in U-bottom microtitre plates. Heat-inactivated serum sample was serially diluted twofold with PBS, pH 7.2 containing BSA and gelatine as described above. The final volume of diluted serum in each well was 50 µl. 25 µl of non-sensitized or sensitized cells were added to each well. The plate was covered, shaken for 5 min and incubated at 37 °C overnight. The degree of haemagglutination was judged by the naked eye and grading was arbitrarily set as – (no haemagglutination reaction), +, ++, +++ and ++++, as the reaction escalated. The highest serum dilution giving ++ haemagglutination was taken as the *Paragonimus* antibody titre. The precision of the IHA was also investigated by performing the test on different days using the same pooled positive serum and the same batch of antigens under identical conditions. Identical results were obtained in all of the tests, indicating that there was no day-to-day variation. Sensitivity, specificity and predictive values were then calculated (Galen 1980).

Results

Purified *Paragonimus heterotremus* antigens prepared by MAb-affinity chromatography were used in IHA for the detection of anti-*P. heterotremus* antibody in sera of 4 groups of subjects and the results are summarized in Table 1. The maximum titre of the healthy control group was 1:128, the minimum titre of the paragonimiasis group, 1:512. The

Table 1 Titre, sensitivity and specificity of IHA using affinity purified *Paragonimus heterotremus* ES antigens

Subjects	Titre (range)	No. positive/total (%)
Paragonimiasis	512–16 384	30/30 (100)
Other parasitosis ¹	2–64	0/92 (0)
Tuberculosis	2–64	0/18 (0)
Healthy control	2–128	0/30 (0)

¹Total of 92 cases, 8 were fascioliasis, 6 were cysticercosis, 8 were gnathostomiasis, 5 were schistosomiasis, 8 were angiostrongyliasis, 18 were trichinosis, 4 were capillariasis, 24 had *Opisthorchis*, 6 had *Opisthorchis* and hookworms, 1 had *Opisthorchis*, hookworm and *Strongyloides*, 2 had *Opisthorchis*, hookworm and minute intestinal flukes and 2 had *Opisthorchis* and *Trichuris*.

cut-off titre was then set at 1:512 and the sensitivity, specificity and positive and negative predictive values calculated at the prevalence at 17.6 % were all 100 %.

Discussion

Our data clearly show that the IHA test using affinity-purified ES antigens we developed gave 100% sensitivity and specificity for detection of antibodies to *P. heterotremus*. The test is thus as good as indirect ELISA for serodiagnosis of human paragonimiasis (Maleewong *et al.* 1997). The IHA test has the advantage over the indirect ELISA in that the results can be read by eye, thus staying away from the expensive microplate spectrophotometer. The IHA test is particularly useful for most diagnostic laboratories in the developing parts of the world where parasitic infections are the cause of public health problems and expensive equipment is not readily available. The stability of sensitized red cells and ease of techniques make it possible to produce the kit and supply it to the laboratories upon request.

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References

- CDC (1976) *A procedural guide to the performance of the serology of toxoplasmosis*. Centers for Disease Control, Atlanta, Georgia.
- Dekumyoy P, Setasuban P, Waikagul J, Yaemput S & Sanguankit S (1995) Human lung fluke *Paragonimus heterotremus*: differentiation of antigenic proteins of adult worms by enzyme-linked immunoelectrotransfer blot technique. *Southeast Asian Journal of Tropical Medicine and Public Health* **26**, 434–438.
- Galen RS (1980) Predictive values and efficiency of laboratory testing. *Pediatric Clinics of North America* **27**, 861–869.
- Indrawati I, Chaicumpa W, Setasuban P & Ruangkunaporn Y (1991) Studies on immunodiagnosis of human paragonimiasis and specific antigen of *Paragonimus heterotremus*. *International Journal for Parasitology* **21**, 395–401.
- Maleewong W, Intapan PM, Priammuenwai M *et al.* (1997) Monoclonal antibodies to *Paragonimus heterotremus* and their potential for diagnosis of paragonimiasis. *American Journal of Tropical Medicine and Hygiene* **56**, 413–417.
- Maleewong W, Pariyanonda S, Wongkham C, Intapan P, Daenseegaew W & Morakote N (1990) Comparison of adult somatic and excretory-secretory antigens in enzyme linked-immunosorbent assay for serodiagnosis of human infection with *Paragonimus heterotremus*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84**, 840–841.
- Maleewong W, Wongkham C, Intapan P, Pariyanonda S & Morakote N (1992) Excretory-secretory antigenic components of *Paragonimus heterotremus* recognized by infected human sera. *Journal of Clinical Microbiology* **30**, 2077–2079.
- Maleewong W, Wongkham C, Pariyanonda S *et al.* (1991) Antigenic components of *Paragonimus heterotremus* recognized by infected human serum. *Parasite Immunology* **13**, 89–93.
- Miyazaki I & Fontan R (1970) Mature *Paragonimus heterotremus* found from a man in Laos. *Japanese Journal of Parasitology* **19**, 109–113.
- Miyazaki I & Harinasuta T (1966) The first cases of human paragonimiasis caused by *Paragonimus heterotremus* Chen *et Hsia* 1964. *Annals of Tropical Medicine and Parasitology* **60**, 509–514.
- Morakote N, Thamasonthi W, Charuchinda K & Khamboonruang C (1984) Prevalence of *Toxoplasma* antibodies in Chiang Mai population. *Southeast Asian Journal of Tropical Medicine and Public Health* **15**, 80–85.
- Pariyanonda S, Maleewong W, Pipitgool V *et al.* (1990) Serodiagnosis of human paragonimiasis caused by *Paragonimus heterotremus*. *Southeast Asian Journal of Tropical Medicine and Public Health* **21**, 103–107.
- Vanijanonta S, Radomyos P, Bunnag D & Harinasuta T (1981) Pulmonary paragonimiasis with expectoration of worm: A case report. *Southeast Asian Journal of Tropical Medicine and Public Health* **12**, 104–106.
- WHO (1995) Control of food borne trematode infections. *World Health Organization Technical Report Series* **849**, 1–157.