

Excretory-Secretory Antigenic Components of *Paragonimus heterotremus* Recognized by Infected Human Sera

W. MALEEWONG,^{1*} C. WONGKHAM,² P. INTAPAN,¹ S. PARIYANONDA,¹ AND N. MORAKOTE³

Department of Parasitology¹ and Department of Biochemistry,² Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, and Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50002,³ Thailand

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Antigenic components of *Paragonimus heterotremus* metabolic products were revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of sera from patients with *P. heterotremus* infection, from patients with other illnesses, and from healthy adults. By SDS-PAGE, it was found that the metabolic products comprised more than eight major polypeptides. Immunoblot analysis revealed 11 components which were strongly recognized by paragonimiasis antisera. These antigenic components had molecular masses ranging from less than 12.3 kDa to 144 kDa. One antigenic band of 31.5 kDa was found to give a consistent reaction with paragonimiasis antisera (97% sensitivity). Of the other patient sera, only sera from patients with *Fasciola* sp. infection reacted with antigenic bands of 56, 38, and 18.5 kDa. The present findings suggest that the 31.5-kDa component is sensitive and specific for the diagnosis of human *P. heterotremus* paragonimiasis.

Human infection with *Paragonimus heterotremus* has been reported in Thailand (17). The clinical diagnosis of paragonimiasis is based primarily on the detection of *Paragonimus* eggs in sputum or feces, whereas serodiagnostic tests play a supportive role in clinical diagnosis. Antibody responses to *P. heterotremus* infection have been demonstrated in human sera by enzyme-linked immunosorbent assay (ELISA) (8, 11). In addition, the immunoblot technique has revealed a specific parasite polypeptide from *P. heterotremus* somatic adult worm extract which reacted with human *P. heterotremus* paragonimiasis antisera (4, 9). However, the antigenic profile of excretory-secretory (ES) products from *P. heterotremus* adult worms has not yet been obtained. In the present study, the ES products were analyzed to obtain this information by using the immunoblot technique and to find their value in diagnosis of human paragonimiasis.

MATERIALS AND METHODS

Parasite and antigen. Adult *P. heterotremus* were obtained from experimentally infected cats which had previously been fed metacercariae isolated from crabs collected from Phitsanulok Province, Thailand, an area where paragonimiasis is endemic. The worms were identified as *P. heterotremus* on the basis of criteria described previously (10). Adult ES antigens were prepared from spent culture medium (protein-free Eagle's basal medium) (GIBCO, Grand Island, N.Y.) in which the worms had been maintained as described by Tuti et al. (16). The spent culture medium was dialyzed against saline and concentrated by vacuum dialysis. The solution was clarified by high-speed centrifugation (10,000 × g, 30 min, 4°C). The supernatant fraction was aliquoted and stored at -40°C. The protein content of antigen was determined by the Folin phenol method (7).

Serum samples. Twenty-nine paragonimiasis antiserum samples were obtained from villagers of Phitsanulok Province (located approximately 400 km north of Bangkok)

whose sputa contained *P. heterotremus* eggs. All of these patients had a history of eating raw crabs from mountain streams in the area of endemic paragonimiasis between 3 and 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, gnathostomiasis, angiostrongyliasis, cysticercosis, thelaziasis, strongyloidiasis, or hookworm infection were obtained from parasitologically confirmed cases. Tuberculosis antisera were obtained from patients whose clinical findings were compatible with pulmonary tuberculosis. Their sputa were positive for acid-fast tubercle bacilli upon culture on Lowenstein-Jensen medium after decontamination by the sodium lauryl sulfate method (1). These sera were also reactive in immunoglobulin G-ELISA against *Mycobacterium tuberculosis* antigen (14). Negative control sera were obtained from 20 healthy adults residing in areas in which paragonimiasis is not endemic. Examination of their stools by the formalin-ether concentration method (2) revealed no evidence of intestinal parasitic infections. Pooled positive and negative reference sera were prepared by combining equal volumes of paragonimiasis antisera or healthy control sera and used for observation of day-to-day variation in the immunoblot analysis.

SDS-PAGE and immunoblotting technique. Components of the metabolic worm products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a 7 to 12% gradient gel prepared by the method of Laemmli (6). A total of 30 µg of protein per lane was loaded onto the gel. After electrophoresis, the resolved polypeptide bands were either revealed by staining with Coomassie brilliant blue stain or electrophoretically transferred to nitrocellulose membranes for immunoblotting. Immunoblotting was conducted as described by Towbin et al. (15). After proteins were transferred onto the nitrocellulose, the membrane was immersed in blocking solution (2% skim milk and 0.1% Tween 20 in 100 mM phosphate-buffered saline, pH 7.4) for 30 min and cut vertically into strips. Each strip was then incubated with each serum sample (diluted 1:100 in blocking solution) for 2

* Corresponding author.

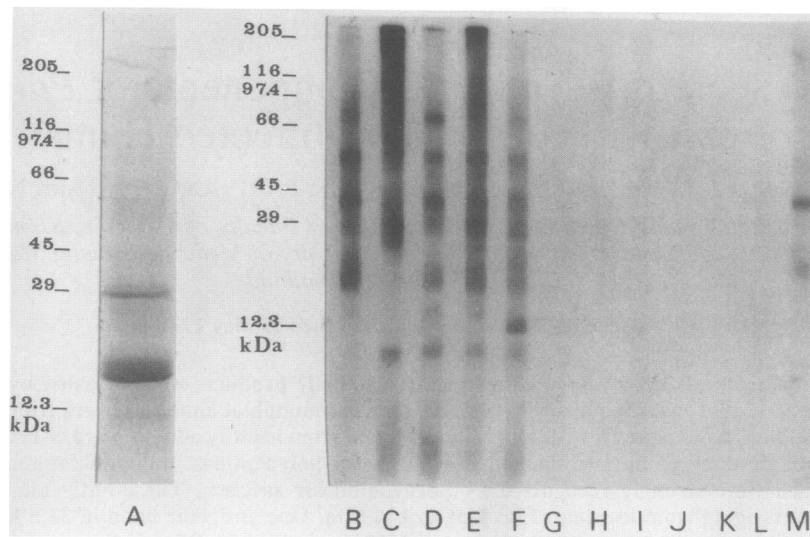


FIG. 1. SDS-PAGE and immunoblot analysis of *P. heterotremus* metabolic products. Lanes: A, Coomassie brilliant blue staining of metabolic products after SDS-PAGE; B to M, autoradiograph of an immunoblot after reaction with paragonimiasis (B to F), healthy control (G), trichinosis (H), gnathostomiasis (I), angiostrongyliasis (J), cysticercosis (K), opisthorchiasis (L), and fascioliasis (M) sera.

h with gentle rocking. The strip was washed five times with fresh blocking solution and subsequently incubated with ^{125}I -labelled protein A (Amersham, Buckinghamshire, United Kingdom). After being washed, the strips were dried and autoradiographed onto diagnostic film (X-OMAT AR; Eastman Kodak Company, Rochester, N.Y.) in a cassette with an intensifying screen at -70°C . The molecular mass was estimated by comparing the mobility of worm antigens with that of a standard protein mixture (Sigma Chemical Co., St. Louis, Mo.) separated on the same gel. The precision of immunoblot analysis was also investigated by performing the test on different days by using the same pooled positive serum, the same batch of antigen, and the same conditions. Identical patterns of bands were obtained from all the tests, indicating that there was no day-to-day variation. Sensitivity, specificity, and predictive values were calculated by the method of Galen (3).

RESULTS

SDS-PAGE analysis and Coomassie brilliant blue staining of *P. heterotremus* ES antigen revealed at least eight major bands with apparent molecular masses ranging from <12.3 kDa to 66 kDa (Fig. 1, lane A). Immunoblotting with paragonimiasis antisera detected 11 major antigenic bands of <12.3 , 12.3, 18.5, 27, 31.5, 38, 56, 89, 91, 123, and 144 kDa

(Fig. 1, lanes B to F). The frequencies of reactivity of each band with sera from the different patient groups are summarized in Table 1. One prominent antigenic band of 31.5 kDa was found to react consistently with most of the sera from patients with paragonimiasis. The specificity of the ES antigen was defined further by comparing the serum reactivities with those of healthy controls, patients with other parasitic infections, and patients with pulmonary tuberculosis (Fig. 1, lanes G to M) (Table 1). Only fascioliasis antisera were found to react with the 56-, 38-, and 18.5-kDa bands.

The sensitivity and specificity of the test for the 31.5-kDa band were 97% and 100%, respectively. Positive and negative predictive values calculated at the prevalence of disease of 32.58% were 100% and 98%, respectively.

DISCUSSION

The present study examined the antigenic components of the metabolic products of *P. heterotremus*. The ES antigens contained a specific 31.5-kDa antigen which reacted mainly with sera from paragonimiasis patients but not with those from patients with other parasitic infections or pulmonary tuberculosis. The results of this study are in agreement with those of our previous report, that the 31.5-kDa component from the somatic antigens of *P. heterotremus* has excellent

TABLE 1. Number of sera which recognized each antigenic component of *P. heterotremus* ES antigens demonstrated by immunoblotting

Serum type	No. of sera tested	No. (%) reacting with component (kDa):										
		144	123	91	89	56	38	31.5	27	18.5	12.3	<12.3
Paragonimiasis	29	1 (3.5)	2 (7)	8 (28)	25 (86)	27 (93)	27 (93)	28 (97)	2 (7)	27 (93)	20 (69)	24 (83)
Fascioliasis	2	0	0	0	0	2 (100)	1 (50)	0	0	2 (100)	0	0
Helminthiasis ^a	33	0	0	0	0	0	0	0	0	0	0	0
Tuberculosis	5	0	0	0	0	0	0	0	0	0	0	0
Healthy control	20	0	0	0	0	0	0	0	0	0	0	0

^a Total of 33 cases; 12 were infected with *Opisthorchis viverrini*, 6 were infected with *Gnathostoma* spp., 5 were infected with *Angiostrongylus* spp., 5 were infected with cysticercus, 1 was infected with a *Thelazia* sp., 1 was infected with a *Strongyloides* sp., and 3 were infected with hookworm.

sensitivity and specificity for diagnosis of human paragonimiasis (9).

Sugiyama et al. (13) reported that the 27-kDa component of *Paragonimus westermani* might be a useful antigen for diagnosis of paragonimiasis. They also showed that the 50-kDa component was recognized by antisera from *P. westermani*-infected hosts but not *Paragonimus miyazakii*-infected hosts. Itoh and Sato (5) showed that the 27-kDa and 33- to 37-kDa components were useful antigens for the diagnosis of paragonimiasis, whereas the 120-kDa component was a candidate for the serodiagnosis of *P. miyazakii* paragonimiasis. In addition, Slemenda et al. (12) demonstrated that the recognition of the 8-kDa component by a patient's serum strongly suggested infection with *Paragonimus* spp. Recently, Indrawati et al. (4) reported that the 35-kDa component of *P. heterotremus* somatic extracts appeared to be a specific antigen. The present study demonstrated great sensitivity and specificity of the 31.5-kDa component from metabolic antigens for the diagnosis of human *P. heterotremus* paragonimiasis. Variation in antigenic bands, reported above by different investigators, may be due to different methods of antigen preparation, antigen preparation from different species, or varying times of blood collection during the course of infection.

The demonstration of a specific 31.5-kDa antigen of *P. heterotremus* would lead to the development of a specific method for diagnosis of human paragonimiasis using simple serological tests, either the ELISA or the latex agglutination test, provided that the specific component of the worms is prepared, through either biochemical processes or recombinant DNA technology.

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REFERENCES

- Engbaek, H. C., B. Vergmann, and M. W. Weis-Bentzon. 1967. The sodium lauryl sulphate method in culturing sputum for mycobacterium. *Scand. J. Respir. Dis.* **48**:268-284.
- Erdman, D. D. 1981. Clinical comparison of ethyl acetate and diethyl ether in the formalin-ether sedimentation technique. *J. Clin. Microbiol.* **14**:483-485.
- Galen, R. S. 1980. Predictive value and efficiency of laboratory testing. *Pediatr. Clin. North Am.* **27**:861-869.
- Indrawati, I., W. Chaicumpa, P. Setasuban, and Y. Ruangkuaporn. 1991. Studies on immunodiagnosis of human paragonimiasis and specific antigen of *Paragonimus heterotremus*. *Int. J. Parasitol.* **21**:395-401.
- Itoh, M., and S. Sato. 1988. An antigenic component for serodiagnosis of *Paragonimus miyazakii*. *Jpn. J. Parasitol.* **37**:347-352.
- Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- Maleewong, W., S. Pariyanonda, C. Wongkham, P. Intapan, W. Daenseegaew, and N. Morakote. 1990. Comparison of adult somatic and excretory-secretory antigens in enzyme-linked immunosorbent assay for serodiagnosis of human infection with *Paragonimus heterotremus*. *Trans. R. Soc. Trop. Med. Hyg.* **84**:840-841.
- Maleewong, W., C. Wongkham, S. Pariyanonda, P. Intapan, V. Pipitgool, W. Daenseegaew, and N. Morakote. 1991. Antigenic components of *Paragonimus heterotremus* recognized by infected human serum. *Parasite Immunol.* **13**:89-93.
- Miyazaki, I., and S. Vajrasthira. 1967. Occurrence of the lung fluke *Paragonimus heterotremus* Chen & Hsia 1964, in Thailand. *J. Parasitol.* **53**:207.
- Pariyanonda, S., W. Maleewong, V. Pipitgool, C. Wongkham, N. Morakote, P. Intapan, B. Thirakharn, and N. Boonphaung. 1990. Serodiagnosis of human paragonimiasis caused by *Paragonimus heterotremus*. *Southeast Asian J. Trop. Med. Public Health* **21**:103-107.
- Slemenda, S. B., S. E. Maddison, E. C. Jong, and D. D. Moore. 1988. Diagnosis of paragonimiasis by immunoblot. *Am. J. Trop. Med. Hyg.* **39**:469-471.
- Sugiyama, H., M. Sugimoto, K. Akasaka, T. Horiuchi, T. Tomimura, and S. Kozaki. 1987. Characterization and localization of *Paragonimus westermani* antigen stimulating antibody formation in both infected cat and rat. *J. Parasitol.* **13**:363-367.
- Thongkrajai, P., V. Lulitanon, and C. Chamnanvanakit. 1989. Improved ELISA with immunosorbent-purified mycobacterial antigen for serodiagnosis of tuberculosis. *J. Med. Microbiol.* **30**:101-104.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
- Tuti, S., S. Vichasri, and S. Sirisingha. 1982. Effect of culture media on production of excretory secretory product and egg output of *Opisthorchis viverrini* in vitro. *J. Parasitol.* **68**:892-897.
- Vanijanonta, S., P. Radomyos, D. Bunnag, and T. Harinasuta. 1981. Pulmonary paragonimiasis with expectoration of worms: a case report. *Southeast Asian J. Trop. Med. Public Health* **12**:104-106.