Evaluation of Human IgG Subclass Antibodies in the Serodiagnosis of Paragonimiasis Heterotremus

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SUMMARRY Immunoglobulin G subclass antibodies (IgG1, IgG2, IgG3, and IgG4) responses to the excretory-secretory antigens of the lung fluke, *Paragonimus heterotremus*, were analyzed using the immunoblotting technique in an attempt to further improve the sensitivity and specificity for serodiagnosis of human paragonimiasis. Serum samples from patients with proven paragonimiasis, from patients with other parasitic infections, pulmonary tuberculosis and from healthy counterparts were analyzed. The results indicate that immunoblotting for the detection of IgG4 antibodies to an excretory-secretory product of *P. heterotremus* of an approximate molecular mass of 31.5 kDa, is the most reliable test. It gives accuracy, sensitivity, specificity, and positive and negative predictive values of 97.6%, 100%, 96.9%, 90% and 100%, respectively.

The lung fluke *Paragonimus* spp. causes paragonimiasis, a harmful lung disease in humans and other animals. It is estimated that over 20 million people are infected worldwide.[1] While *Paragonimus westermani* is the most common species, *P. heterotremus* is the main etiological agent of human paragonimiasis in China, Laos, and Thailand.[2]

Currently, the diagnosis of paragonimiasis is based on the demonstration of *Paragonimus* eggs in feces, or sputa, or both.[3] However, the microscopic examination of the samples for the parasite's eggs is laborious and time-consuming, and reliable only in the hands of experienced personnel. Furthermore, it is sometimes subject to bias and confusion with other digenean flukes. In addition, the clinical symptoms of paragonimiasis are frequently confused with non-parasitic respiratory diseases, such as pulmonary tuberculosis. The immunological tests have become the most practical, useful and dependable assays in extrapulmonary paragonimiasis or during the lag period between initial infection with metacercariae and sexual maturation of the egg laying adult. Immunological techniques have been used with varying success to diagnose *P. heterotremus* infections by detecting specific antibodies in the sera of infected

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individuals.[4-7] The 31.5 kDa as well as the 35 kDa antigens from *P. heterotremus* adult worms are the most specific antigens for the diagnosis of human paragonimiais.[4-6]

The specificity and sensitivity of the serodiagnosis of filariasis,[8-10] strongyloidiasis,[11] chronic schistosomiasis,[12, 13] trichuriasis,[14] hookworm infection,[15] angiostrongyliasis,[16] and gnathostomiasis[17] have both increased through the detection of IgG subclass antibodies to antigens of these parasites. However, little is known about the antigenic components of *P. heterotremus* recognized by the different IgG subclasses in infected humans. Here, we investigated the different subclasses of human IgG antibodies contributing to the immune recognition of a molecule with an apparent molecular mass of 31.5 kDa from an adult *P. heterotremus* excretory-secretory (ES) antigen. The aim was to determine whether the detection of any subclass of IgG antibodies could be used to improve the sensitivity and specificity of this immunodiagnostic technique.

MATERIALS AND METHODS

Parasite collection and preparation of ES antigen

Adult *P. heterotremus* worms were obtained from the lungs of experimentally infected cats and used for the preparation of ES antigen. The worms were identified as *P. heterotremus* as previously described.[18] The ES antigen of *P. heterotremus* adult worms was prepared from RPMI 1640 culture medium containing 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 0.1 mM N-tosyl-L-phenylalanine-chloromethyl ketone (TPCK) used for maintaining the worms.[6] The adult worms were incubated six times for 2 hours per incubation at 37°C to avoid the effect of a protease enzyme that can degrade the ES antigen. The spent medium was concentrated by ultrafiltration using an Amicon YM 3 membrane filters (Grace & Co., MA) and dialyzed against distilled water containing 0.1 mM PMSF and 0.1 mM TPCK. The solution was clarified by centrifugation at 10,000 x *g* for 30 minutes, aliquoted and stored at - 40°C. The protein content was determined by the Folin phenol method.[19]

Human sera

Human sera were obtained from 4 groups of subjects. Group 1 comprised 18 patients with proven paragonimiasis heterotremus 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. Group 2 comprised 45 parasitologically confirmed cases of opisthorchiasis, strongyloidiasis, fascioliasis, cysticercosis, capillariasis, gnathostomiasis, angiostrongyliasis, and trichinosis. Group 3 comprised 10 patients with tuberculosis whose clinical findings were compatible with pulmonary tuberculosis and their sputa were positive for acid fast tubercle bacilli. Group 4 was made up of 10 healthy Thai adults whose stool samples, collected at the same time as serum sampling, were examined using the formalin-ethyl acetate concentration method[20] and gave no evidence of intestinal parasitic infections.

Pooled positive or negative reference sera were prepared by combining equal volumes of paragonimiasis antisera or healthy adult sera, and were used for observation of day-to-day variations in the immunoblotting analysis. The study protocol was approved by the Human Research Ethics Committee of Khon Kaen University. Informed consent was obtained from the study subjects using a standard approved procedure.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

The *P. heterotremus* ES antigen was separately resolved by SDS-PAGE under reducing conditions on a 10 to 15% gradient gel prepared using the method of Laemmli.[21] Antigen containing 360 μg protein per lane of 7 cm width was loaded onto the gel. After electrophoresis, the resolved polypeptides were electrophoretically transferred to the nitrocellulose membrane for immunoblotting.[22] The antigen-blotted nitrocellulose membrane was immersed in a blocking solution (1% skimmed milk in 0.1 M phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20® [Sigma, St. Louis, MO], PBS-T) and cut vertically into strips of 0.4 x 5.5 cm. One strip was incubated with one serum sample (diluted 1:50 in the blocking solution) for 2 hours at 25°C with gentle rocking, washed five times with blocking solution

and then incubated for 2 hours at 25°C with peroxidase conjugated monoclonal anti-human IgGi (i = 1, 2, 3 or 4) subclass (Zymed Laboratories, Inc., South San Francisco, CA) in a blocking solution diluted 1:500 for IgG1, IgG2 and IgG3 and diluted 1:10,000 for IgG4. After washing, the strips were incubated in diaminobenzidine (Sigma) in 50 mM Tris-HCl, pH 7.6. The blot was developed at 25°C with agitation until dark brown bands appeared. The strips were then washed with distilled water and air-dried. The precision of the immunoblot analysis was investigated by performing the test on different days using the same pooled positive serum, the same pooled negative serum and the same batch of antigen under the same conditions. Identical patterns of bands were obtained from all tests, indicating that there was no day-to-day variation.

Data analysis

The relative molecular masses of the antigens recognized in the serum samples, were determined using a logarithmic plot of migration of a set of molecular mass standards included in every assay. The diagnostic accuracy, sensitivity, specificity and predictive values were calculated as previously described.[23] These values were calculated and expressed as: accuracy = ([no. of true positives + no. of true negatives]/[no. of true positives + no. of false positives + no. of true negatives + no. of false negatives]) x 100; sensitivity = (no. of true positives/[no. of true positives + no. of false negatives]) x 100; specificity = (no. of true negatives/[no. of true negatives + no. of false positives]) x 100; positive predictive value = (no. of true positives/[no. of true positives + no. of false positives]) x 100; negative predictive value = (no. of true negatives/[no. of true negatives + no. of false negatives]) x 100; true negative = number of control samples (other parasitosis, pulmonary tuberculosis and healthy controls) that did not react with the 31.5 kDa antigen; true positive = number of proven paragonimiasis samples that reacted with the 31.5 kDa antigen; false positive = number of control samples that reacted with the 31.5 kDa antigen; false negative = number of proven paragonimiasis samples that did not react with the 31.5 kDa antigen.

RESULTS

The immunoblotting results with the sera from the 4 groups of the samples are shown in Table 1 and Figs. 1 to 4. The antigen recognition patterns for IgG1, IgG2, IgG3 and IgG4 were different with molecular masses scattering from less than 17.8 to less than 48.5 kDa. In the proven paragonimiasis group (Group 1), all of the 18 sera had IgG4, 12 had IgG3, 16 had IgG2 and 15 had IgG1 antibodies to the

Table 1 Summary of IgG subclass antibodies against the 31.5 kDa component of *Paragonimus heterotremus* antigen demonstrated by immunoblotting

Serum type (group)	No. (%) reacting with 31.5 kDa component			
	IgG_1	IgG_2	IgG_3	IgG_4
Paragonimiasis (1)	15/18 (83.3)	16/18 (88.9)	12/18 (66.7)	18/18 (100)
Opisthorchiasis (2)	2/10 (20)	0/10 (0)	0/10 (0)	0/10 (0)
Strongyloidiasis (2)	0/10 (0)	1/10 (10)	0/10 (0)	0/10 (0)
Fascioliasis (2)	5/10 (50)	7/10 (70)	6/10 (60)	2/10 (20)
Cysticercosis (2)	0/3 (0)	1/3 (33.3)	0/3 (0)	0/3 (0)
Capillariasis (2)	1/3 (33.3)	2/3 (66.7)	1/3 (33.3)	0/3 (0)
Gnathostomiasis (2)	0/3 (0)	3/3 (100)	0/3 (0)	0/3 (0)
Angiostrongyliasis (2)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
Trichinosis (2)	2/3 (66.7)	3/3 (100)	0/3 (0)	0/3 (0)
Pulmonary tuberculosis (3)	0/10 (0)	4/10 (40)	4/10 (40)	0/10 (0)
Healthy control (4)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)

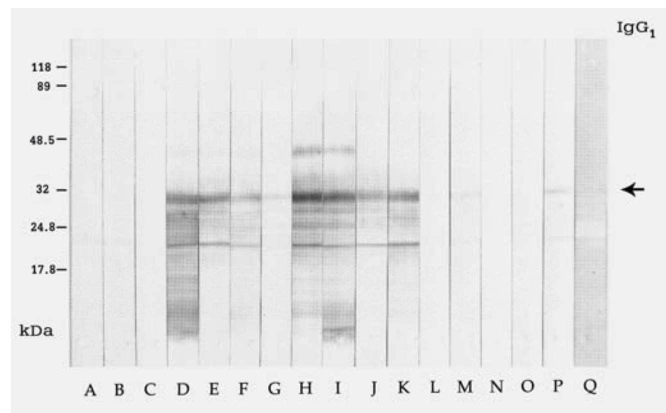

Fig. 1 Representative immunoblot patterns showing IgG1 antibodies reactive to *Paragonimus heterotremus* excretory-secretory antigens. Blots reacted with pooled negative reference (A), healthy control (B-C), pooled positive reference (D), proven paragonimiasis (E-I), fascioliasis (J-K), pulmonary tuberculosis (L), opisthorchiasis (M), strongyloidiasis (N), cysticercosis (O), capillariasis (P), and gnathostomiasis (Q) sera. The position of the diagnostic 31.5 kDa band is marked on the left (arrow).

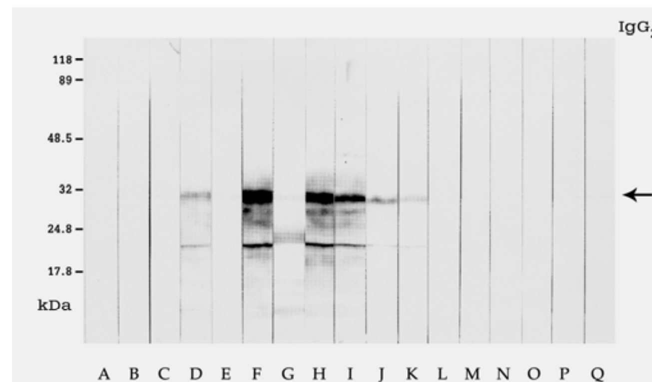

Fig. 2 Representative immunoblot patterns showing IgG2 antibodies reactive to *Paragonimus heterotremus* excretory-secretory antigens. Blots reacted with sera from pooled negative reference (A), healthy control (B-C), pooled positive reference (D), proven paragonimiasis (E-I), fascioliasis (J-K), pulmonary tuberculosis (L), opisthorchiasis (M), strongyloidiasis (N), cysticercosis (O), capillariasis (P), and gnathostomiasis (Q) sera. The position of the diagnostic 31.5 kDa band is marked on the left (arrow).

31.5 kDa antigen. In the other parasitosis group (Group 2), the reactions of the IgG subclass antibodies to the 31.5 kDa antigen varied widely (Table 1). In the pulmonary tuberculosis group (Group 3), 4 serum samples had IgG2 antibodies and 4 serum samples had IgG3 antibodies to the 31.5 kDa antigen. No serum from the healthy control group (Group 4) showed any reaction between any subclass of antibodies to the 31.5 kDa antigen. Table 2 shows the diagnostic values for the detection of the four IgG subclass antibodies to the 31.5 kDa antigen when they were used as the markers for diagnosis of human paragonimiasis. The IgG4 subclass antibody demonstrated the highest diagnostic values. However, two fascioliasis sera reacted faintly against the 31.5 kDa antigen with the IgG4-immunoblotting. The accuracy, sensitivity, specificity and positive and negative predictive values of the IgG4 test were 97.6%, 100%, 96.9%, 90% and 100%, respectively.

DISCUSSION

The human immune responses to parasitic infections exhibit different distributions of IgG subclass antibodies between infected groups.[24, 25] The genetic background of the infected individual as well

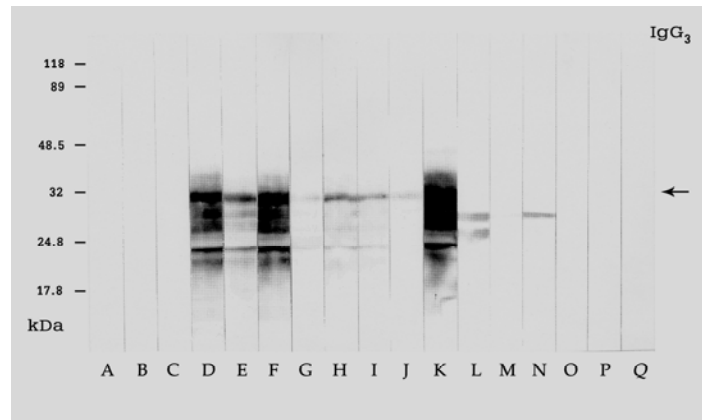

Fig. 3 Representative immunoblot patterns showing IgG3 antibodies reactive to *Paragonimus heterotremus* excretory-secretory antigens. Blots reacted with sera from pooled negative reference (A), healthy control (B-C), pooled positive reference (D), proven paragonimiasis (E-I), fascioliasis (J-K), pulmonary tuberculosis (L), opisthorchiasis (M), strongyloidiasis (N), cysticercosis (O), capillariasis (P), and gnathostomiasis (Q) sera. The position of the diagnostic 31.5 kDa band is marked on the left (arrow).

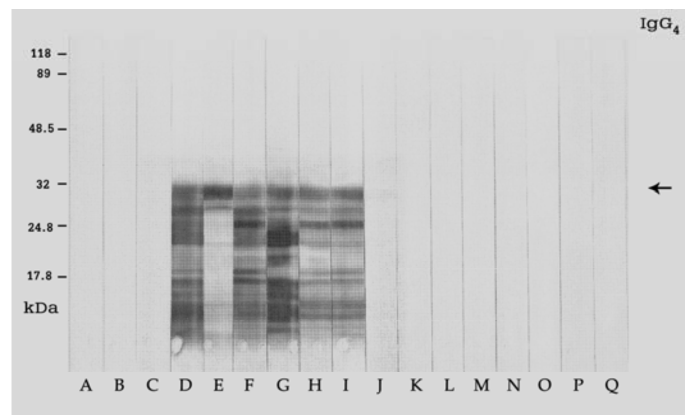

Fig. 4 Representative immunoblot patterns showing IgG4 antibodies reactive to *Paragonimus heterotremus* excretory-secretory antigens. Blots reacted with sera from pooled negative reference (A), healthy control (B-C), pooled positive reference (D), proven paragonimiasis (E-I), fascioliasis (J-K), pulmonary tuberculosis (L), opisthorchiasis (M), strongyloidiasis (N), cysticercosis (O), capillariasis (P), and gnathostomiasis (Q) sera. The position of the diagnostic 31.5 kDa band is marked on the left (arrow).

as the intrinsic properties of the antigen itself play the major role in determining the subclass of antibody response.[26] We here describe various antigenic patterns of a *P. heterotremus* ES product, recognized with sera from patients with proven paragonimiasis, from patients with other parasitic infections, from patients with pulmonary tuberculosis and from healthy volunteers using immunoblotting and subclass-specific reagents. Differentiation of antigenic recognition with any subclass of IgG antibodies could be used to improve the sensitivity and specificity of the immunodiagnostic technique.[15,16]

Paragonimiasis, caused by *P. heterotremus*, is an important endemic disease in southeastern Asia. To ensure proper control and perhaps to eradicate paragonimiasis, regular prevalence surveys are necessary. Thus an accurate, rapid, sensitive, and specific diagnostic method is essential for epidemiological studies and/or clinical diagnosis. Indrawati *et al.*[4]

Table 2 Diagnostic values for the detection of various IgG subclass antibodies against the 31.5 kDa component by immunoblotting

IgG subclass	Sensitivity (%)	Specificity (%)	Accuracy (%)	Positive predictive value (%)	Negative predictive value (%)
IgG_1	83.3	84.6	84.3	60	94.8
IgG_2	88.9	67.7	72.3	43.2	95.7
IgG_3	66.7	83.1	79.5	52.2	90
IgG_4	100	96.9	97.6	90	100

reported that the 35 kDa component of *P. heterotremus* somatic extract appeared to be a specific antigen of the parasite. Other reports[5, 6] showed that the somatic and ES antigens containing the specific 31.5 kDa antigen reacted specifically with sera from paragonimiasis but not with sera from patients with other parasitic infections or pulmonary tuberculosis.

The present study found that an immunoblot with IgG4 was more distinct than with the other subclasses and the intensities were greater. In addition, the detection of IgG4 antibodies to the 31.5 kDa antigen in human sera gave the highest diagnostic values among the IgG subclasses. This make the IgG4 is the subclass of choice to be assessed in an immunological test for the diagnosis of human paragonimiasis as well as for use in prevalence determination.

However, a faint reaction was demonstrated against the 31.5 kDa in 2 of 10 sera from fascioliasis patients whereas no cross reaction was shown in the previous report.[6] This result may be explained by the low numbers of samples (2) used in this previous report[6] while more serum samples were tested in the present study, which increased the possibility of cross-reactions. Possible explanations for the cross reactions may be that the patients with fascioliasis were previously infected with *P. heterotremus* and/or that the 31.5 kDa shares some epitopes with the *Fasciola* trematodes, possibly shared by several parasites.[27, 28] However, this cross reactivity does not pose a problem in the clinical setting because infections with the liver fluke, *Fasciola*, normally present different clinical features.

In addition, the finding on IgG1, IgG2 and IgG3 subclass antibodies for the cross reactivity which did not agree with the earlier finding[5, 6] possible be explained by the different conditions of immunoblotting. The previous reports have used ^{125}I-labeled protein A as the probe for recognition of specific IgG antibody.[5,6] Different binding behavior of IgG subclass antibodies to protein A[29] may present different patterns of cross reactivity between previous reports[5, 6] with the present study. It is concluded that detection of the specific IgG4 antibodies to the 31.5 kDa ES antigen of *P. heterotremus* is most reliable test for the serodiagnosis of paragonimiasis hetertremus.

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