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BRIEF COMMUNICATION

Evaluation of IgG4 Subclass Antibody Detection by Peptide-Based ELISA for the Diagnosis of Human Paragonimiasis Heterotrema

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Abstract: A synthetic peptide was prepared based on the antigenic region of *Paragonimus westermani* pre-procathepsin L, and its applicability for immunodiagnosis for human paragonimiasis (due to *Paragonimus heterotremus*) was tested using an ELISA to detect IgG4 antibodies in the sera of patients. Sera from other helminthiases, tuberculosis, and healthy volunteers were used as the references. This peptide-based assay system gave sensitivity, specificity, accuracy, and positive and negative predictive values of 100%, 94.6%, 96.2%, 100%, and 88.9%, respectively. Cross reactivity was frequently seen against the sera of fascioliasis (75%) and hookworm infections (50%). Since differential diagnosis between paragonimiasis and fascioliasis can be easily done by clinical presentation and fascioliasis serology, this cross reaction is not a serious problem. Sera from patients with other parasitoses (0-25%) rarely responded to this synthetic antigen. This synthetic peptide antigen seems to be useful for development of a standardized diagnostic system for paragonimiasis.

Key words: *Paragonimus heterotremus*, paragonimiasis, serodiagnosis, peptide-based ELISA

The lung flukes, *Paragonimus* species, cause paragonimiasis in humans and animals [1-3]. It is estimated that over 20 million people are infected worldwide [4] and approximately 292.8 million people are at risk of infection [5]. While *Paragonimus westermani* is the most important human pathogen in China, Korea, and Japan [1-3,6], *Paragonimus heterotremus* is the main etiological agent of human paragonimiasis in southern China, Southeast Asia (including Thailand and Vietnam), and India [1-3].

Diagnosis of paragonimiasis relies on demonstration of *Paragonimus* eggs in the feces, sputum, or both [3]. However, microscopic detection of *Paragonimus* eggs is not very sensitive because of irregular egg production and difficulties of processing sputum and fecal specimens and requirement for experienced microscopists. Furthermore, eggs are not detectable in ectopic or prepatent paragonimiasis cases. For physicians, clinical symptoms of paragonimiasis are frequently confused with those of non-parasitic respiratory diseases, such as pulmonary tuberculosis and lung cancer. Supportive evidence of immunological tests is, therefore, essential for clinical diagnosis of paragonimiasis. For *P. heterotremus* infection, detection of specific antibodies in the sera of patients has been reported [7-10]. By immunoblot analysis, 31.5 kDa or 35 kDa antigens from *P. heterotremus* adult worms are supposed to be the most specific antigens with the highest diagnostic value [7-9].

Detection of particular immunoglobulin subclasses often improves the development and interpretation of serological assays. Analysis of IgG subclass antibodies can increase specificity and sensitivity of the immunological assay for diagnosis of paragonimiasis [11,12]. Development of serological diagnosis, however, is hampered by the limited availability of antigens as they should be prepared from live worms, which were collected from the field or prepared by experimental infection in laboratory animals. Moreover, varying preparation methods

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for antigens makes inter-laboratory standardization difficult. Thus, the use of a synthetic peptide as a diagnostic antigen is a good alternative and has been studied for some parasitic infections. The peptide-based ELISA for detection of IgG4-specific antibodies improved the diagnostic value for neurocysticercosis [13] and fascioliasis [14]. In the present study, the diagnostic value of IgG4-specific antibody detection by peptide-based ELISA was explored for human paragonimiasis due to *P. heterotremus*.

A synthetic peptide of the antigenic region of *P. westermani* pre-procathepsin L, corresponding to amino acids 216-227 (12 amino acids) of the enzymatic part (GenBank accession no. AAB93494), was synthesized as a carboxamide at the C-terminus and acetyl at the N-terminus: acetyl-AKIDDSIVLEKN-amide by the Mimotopes Pty Ltd (Victoria, Australia), with optimum prediction scores of epitope (>0.9) [15] and hydrophilicity (~1.3) [16]. The amino acid sequence was subjected and analyzed with software available online at http://tools.immuneepitope.org/tools/bcell/iedb_input.

Sera were obtained from serum bank of the Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Each serum was aliquoted and stored at -70˚C until used. The study protocol was approved by the Human Research Ethics Committee of Khon Kaen University (HE5000225). Informed consent was obtained from all adult participants or the parents or legal guardians. Sera were from parasitologically confirmed cases of paragonimiasis (*P. heterotremus*) (n=16), fascioliasis (n=9), opisthorchiasis (n=5), strongyloidiasis (n=10), capillariasis philippinensis (n=20), gnathostomiasis (n=18), angiostrongyliasis (n=6), taeniasis (n=6), cysticercosis (n=8), hookworm infections (n=4), trichinosis (n=6), bancroftian filariasis (n=1), falciparum malaria (n=1), and vivax malaria (n=1). Five sera from patients with pulmonary tuberculosis were also tested. Negative control sera were obtained from 37 healthy adults whose stool examinations were negative by the formalin-ether concentration method for parasite eggs at the time of the blood collection. Pooled positive and negative sera were prepared by combining equal volumes of proven paragonimiasis and healthy control sera, respectively. One serum from a paragonimiasis westermani case was provided by Prof. Yoon Kong, Department of Molecular Parasitology, Sungkyunkwan University School of Medicine, Korea.

The method was performed as previously described [14] with some modifications. Each well of an ELISA plate was coated with 1 µg of peptide in 0.1 ml of 0.01 M phosphate buffer (PBS, pH 7.5) at 4˚C overnight. The wells were washed 3 times with 10 mM PBS, pH 7.5 containing 0.05% Tween 20 (PBS/T) and subsequently blocked with 3% BSA in PBS/T for 1 hr at room temperature. After washing with PBS/T, the wells were incubated at 37˚C for 1 hr with 0.1 ml of human sera diluted 1:50 with 1% BSA in PBS/T. After another washing with PBS/T, a peroxidase-conjugated goat anti-human IgG4 subclass antibody (Zymed, South San Francisco, California, USA) diluted 1:500 with 1% BSA in PBS/T was added and incubated at 37˚C for 1 hr. The wells were then washed with PBS/T and 0.1 ml of reaction mixture (substrate buffer pH 5.0; 0.1 M citric acid and 0.2 M phosphate, 40 mg% orthophenylene diamine and 0.01% hydrogen peroxide) was added and the plate was left standing at room temperature for 30 min. The reaction was stopped with 0.05 ml of 8 N H_2SO_4 and the optical density (OD) was measured at 492 nm using a microplate ELISA reader (Tecan, Salzburg, Austria). Optimum conditions were obtained from titration with pooled positive paragonimiasis (*P. heterotremus*) and pooled negative sera. To ensure the antigenicity of the synthetic peptide, triplicate ELISA testing was performed with paragonimiasis westermani serum and the OD value was 1.37 ± 0.11.

Assay reproducibility was evaluated by calculating the coefficient of variation of the positive control tested in each run, and the test was shown to be reproducible, with calculated inter-assay coefficient variation of 5.6%. Differences in the results between the 2 groups were analyzed by the Mann-Whitney Rank Sum test or Student's *t*-test as appropriate, with a *P*-value less than 0.05 being set as significant using the statistical software Sigma Stat (San Rafael, California, USA). The mean OD value+2.5 SD (0.316) of the healthy group sera optimized by receiver operating characteristic (ROC) curve analysis was used as the lower limit of positivity. The diagnostic sensitivity, specificity, accuracy, and predictive values were calculated and expressed using the method of Galen [17].

The results of detection of specific IgG4 subclass antibodies against synthetic peptides by ELISA in all serum samples, the mean OD values (and SD) and the number of positive sera in each group are shown in Fig. 1. The mean OD value of the proven paragonimiasis group was significantly higher than that of the other parasitic disease groups except for the fascioliasis group. To determine the values of usefulness, the specificity was calculated against healthy and tuberculosis sera only. The sensitivity, specificity, accuracy, and positive and negative predictive values of the ELISA using peptide antigen were 100%, 94.6%, 96.2%, 100%, and 88.9%, respectively. Levels of cross

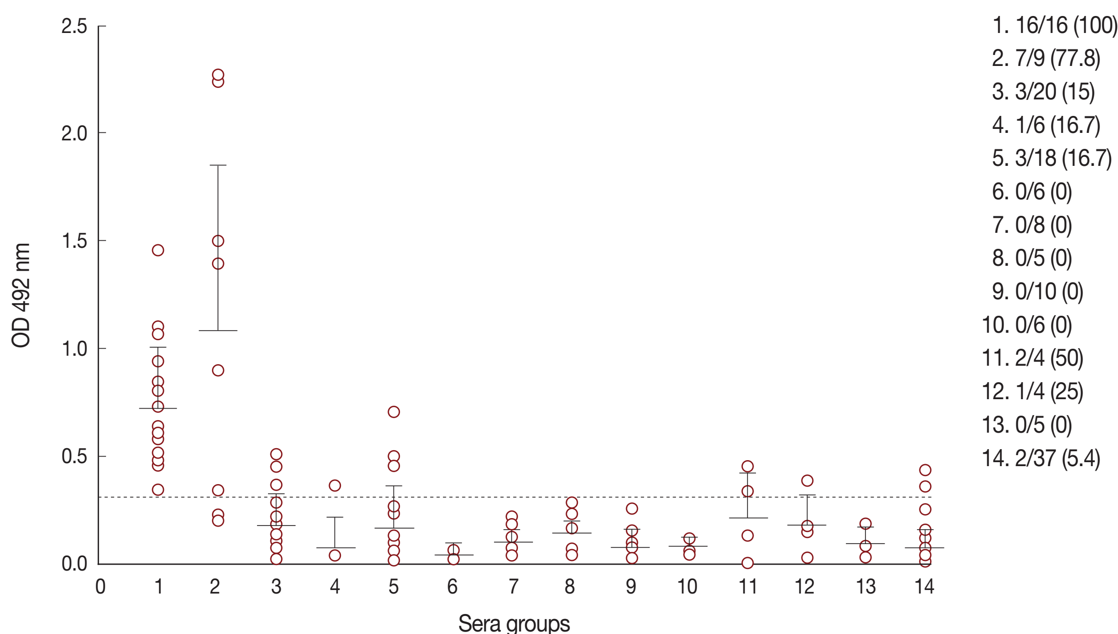

Fig. 1. Absorbance of ELISA (492 nm) using peptide antigen for detection of specific IgG4 antibodies in sera from cases with proven paragonimiasis, parasitic diseases other than paragonimiasis, pulmonary tuberculosis, and healthy controls. Sera groups (mean OD ± SD): 1 = paragonimiasis (0.713 ± 0.309); 2 = fascioliasis (1.047 ± 0.841); 3 = capillariasis (0.199 ± 0.135); 4 = angiostrongyliasis (0.095 ± 0.130); 5 = gnathostomiasis (0.165 ± 0.198); 6 = taeniasis (0.052 ± 0.018); 7 = neurocysticercosis (0.150 ± 0.064); 8 = opisthorchiasis (0.158 ± 0.102); 9 = strongyloidiasis (0.081 ± 0.074); 10 = trichinosis (0.082 ± 0.044); 11 = hookworm infections (0.230 ± 0.198); 12 = other parasitoses (0.182 ± 0.150); 13 = pulmonary tuberculosis (0.101 ± 0.079); 14 = healthy controls (0.085 ± 0.092). Numbers in the box indicate no. of positive/total (%). Dash-line indicates the cut-off optical density (OD) value = 0.316.

reactions against fascioliasis (75%) and hookworm infections (50%) were found to be unacceptably high. Sera from patients with other parasitoses reacted relatively rarely (0-25%) against the peptide (Fig. 1).

One of the major problems of serodiagnosis for parasitic infections is the limited availability of parasite antigens. To prepare *Paragonimus* antigens, adult worms should be collected from naturally or experimentally infected animals. Synthetic peptide is a good alternative antigen and has recently been used for diagnosis of human echinococcosis [18]. In the present study, synthetic peptide based on an antigenic region of *P. westermani* pre-procathepsin L was prepared and used as antigen for detection of IgG4 antibodies. Although the antigenic epitope was constructed from peptide sequence of the *P. westermani* pre-procathepsin L, the assay reported here revealed high sensitivity and positive predictive value (100%) for diagnosis of human *P. heterotremus* infection. The peptide epitope therefore seems to be shared by both *Pargonimus* species. However, high rates of positivity in fascioliasis (75%) and hookworm infections (50%) deserve further attention. Since clinical signs and symptoms of pulmonary paragonimiasis are different from fascioliasis, clinical presentation might help to distinguish between the diseases, or serum should be additionally tested by peptide-based ELISA for fascioliasis as only 8% of paragonimiasis sera were positive [19].

In conclusion, the present study demonstrated that synthetic peptide antigens in IgG4-ELISA can be used for diagnosis of pulmonary paragonimiasis due to *P. heterotremus*.

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