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Applicability of ABC-ELISA and Protein A-ELISA in serological diagnosis of cysticercosis

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Abstract: Specific antibody test in serum and cerebrosinal fluid (CSF) is still the main mode of serological diagnosis of cystiercosis. Of different techniques of antibody test, enzyme-linked immunosorbent assay (micro-ELISA) has widely been applied. This study was undertaken to observe whether diagnostic capability can be improved by applying more sensitive techniques such as Protein A-ELISA and avidin biotin complex ELISA (ABC-ELISA). When evaluated using 115 sera of human cysticercosis, the antibody positive rates were not significantly improved in Protein A-ELISA (82.6%) and in ABC-ELISA (86.1%) than in micro-ELISA (81.7%). The specificities, evaluated in 165 sera from other diseases and normal controls, were significantly improved (88.5% by micro-ELISA, 93.3% by Protein A-ELISA and 93.8% by ABC-ELISA). Antibody levels (absorbance, abs.) in individual serum were correlated well (r = 0.83~0.86) each other. An actual benefit of Protein A-ELISA and ABC-ELISA was that they needed smaller amount of test sample.

Key words: *Taenia solium* metacestode, cysticercosis, serodiagnosis

INTRODUCTION

Human cysticercosis is a helminthic disease caused by systemic infection of *Taenia solium* metacestodes. In many developing countries where *T. solium* infections are prevalent, the possibility of neurocysticercosis should be considered especially in neurologic patients (Flisser *et al.*, 1980; Nash and Neva, 1984). Definitive diagnosis of cysticercosis is made when biopsy exhibited the larva. Nowadays, however, diagnostic and therapeutic biopsy of the brain lesion is undertaken limitedly because imaging diagnosis of computerized tomography (CT) or magnetic resonance (MR) are available together with effective anthelmintics. In addition, serologic tests for specific antibody have widely been employed (Flisser and Larralde, 1986; Cho *et al.*, 1986; Chang *et al.*, 1988) in differentiating the neurocysticercosis especially in patients with ambiguous imaging findings.

Serological diagnosis of cysticercosis has been done by antibody test. Sensitive and specific antigen used in the antibody test is cystic fluid (CF) of *T. solium* metacestodes (Choi *et al.*, 1986; Larralde *et al.*, 1986; Bailey *et al.*, 1988). Though CF is used as an antigen, the sensitivity of the specific antibody test by micro-ELISA is 70-80% in serum and 80-85% in CSF samples. When both serum and CSF are tested, the sensitivity raised to 90% (Cho *et al.*, 1986). The false negative reactions are occurring when all the infected worms are completely calcified or when a few worms are infected (Chang *et al.*, 1988; Wilson *et al.*, 1991). It seems necessary, therefore, to observe whether the low antibody levels in such patients can be detected by more sensitive techniques of the serology.

In this study, we applied more sensitive

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techniques of ABC-ELISA and Protein A-ELISA in detecting the specific antibody to *T. solium* metacestodes in clinical patients of neurocysticercosis. Our objective was to find out the merits of these antibody detecting enzyme immunoassays for cysticercosis.

MATERIALS AND METHODS

1. Cystic fluid of *Taenia solium* metacestodes

Cystic fluid (CF) was obtained as described by Choi *et al.* (1986). Protein content was 3.3 mg/ml when measured by Lowry *et al.* (1951). CF was stored at -70°C until used.

2. Tested human sera

Neurocysticercosis: A total of 115 neurocysticercosis patients sera was selected randomly from sera file of Department of Parasitology, Chung-Ang University. Their clinical manifestations included seizure, headache, nausea, vomiting and motor /sensory weakness with/without focal neurologic deficits. They were diagnosed by neurosurgery or by combination of specific-antibody tests by micro-ELISA in serum and /or cerebrospinal fluid (CSF) and characteristic CT/MRI findings (Cho *et al.*, 1986; Chang *et al.*, 1988).

Sparganosis: Twentyfour sparganosis sera were selected from our serum file of surgically confirmed patients, and patients with characteristic CT findings with positive anti-sparganum (IgG) antibody titers by ELISA (Chang *et al.*, 1987).

***T. saginata* infections:** A total of 7 sera of *Taenia saginata* infections which were confirmed by recovery of proglottids in the stool was used in this study.

Paragonimiasis: A total of 29 sera from paragonimiasis patients which were selected randomly from our patients sera file was subjected to the study. The patients manifested mainly chest discomfort, blood-tinged sputum, cough *etc* with abnomal chest X-ray findings (Shim *et al.*, 1991). Eggs of *Paragonimus* were detected in 6 patients. The patients were diagnosed by positive reactions to anti-*Paragonimus* IgG antibody test by ELISA (Cho *et al.*, 1981).

Clonorchiasis: Out of positive reactors of anti-*Clonorchis* IgG antibody in serum, 28 randomly selected patients sera were examined. The patients were ill of biliary tract diseases and also positive reactors to intradermal test. Fifteen patients were confirmed by stool examination.

Patients of other neurologic diseases: A total of 61 neurologic patients sera was subjected to the test. They were diagnosed medically or surgically as other neurologic disease of astrocytoma, glioblastoma multiforme, brain abscess, vascular diseases *etc.*

Normal control: A total of 16 medical students of Chung-Ang University was examined. They denied exposure to any possible source of parasitic infections such as freshwater fish, soy-bean sauce soaked crabs, raw or undercooked pork, beaf and snakes.

3. ELISA techniques

Three techniques of ELISA, which are different in secondary antibody and their enzyme conjugates, were applied in detecting the specific (IgG) antibody in cysticercosis.

Micro-ELISA: The methods described by Cho *et al.* (1986) were followed as shown in Table 1. In conducting test, a positive and a negative reference serum were tested every time for standardization.

Protein A-ELISA: The general procedures, described in Zymed Lab. (1990) were followed. After the chequerboard titrations, CF diluted in carbonate buffer (pH 9.6) (2.5 μg/ml of protein) were coated to microtiter plate (Costar, U.S.A.) overnight at 4°C. After washing the wells with phosphate buffered saline/Tween 20 (PBS/T, pH 7.4) 3 times, each serum, diluted at 1:200 in PBS/T (pH 7.4) was reacted for 2 hours at 36°C. After washing, 1:2,000 diluted protein A-peroxidase (EIA grade, Zymed, U.S.A.) were reacted for 2 hours at 36°C. Finally, the reactions were colored by 1:2,500 diluted 2,2-azino-di (3-ethylbenzthiazoline) sulfonic acid (ABTS) in citrate buffer (0.1 M, pH 4.2) containing 0.03% H_2O_2 for 15 minutes. The reaction was stopped by adding 2 mM NaN_3. Abs. was read at 415 nm using Microplate Reader (Bio-Rad M 3550, U.S.A.). The tests were also standardized using a positive and negative reference sera.

Table 1. Procedures and conditions of 3 different ELISA techniques employed in this study

	Micro-ELISA	Protein A-ELISA	ABC-ELISA
Protein content in coating antigen	2.5 μg/ml	2.5 μg/ml	2.5 μg/ml
Coating condition	4°C, overnight	4°C, overnight	4°C, overnight
Serum dilution in PBS/T	1:100	1:200	1:10,000
Reaction condition	36°C, 2 hours	36°C, 2 hours	36°C, 1 hour
Conjugate	HRP*-antihuman IgG	HRP-Protein A	Biotin-antihuman IgG
dilution in PBS/T	1:1,000	1:2,000	1:100,000
Reaction condition	36°C, 2 hours	36°C, 2 hours	36°C, 1 hour
			HRP-Avidin, 1:40,000
			36°C, 1 hour
Chromogen	OPD in dist. water,	ABTS in citrate buffer (pH 4.2)	ABTS in citrate buffer (pH 4.2)
Reaction condition	24 minutes	15 minutes	15 minutes
Reading abs.	490 nm	415 nm	415 nm
Cut-off abs.	0.18	0.18 or 0.10	0.18 or 0.10

*HRP: Horseradish peroxidase

Table 2. Results of specific-antibody tests by 3 different techniques of ELISA in each category of patients

Disease category	No. of patients	Absorbance (Mean ± S.D.) in		
		Micro-ELISA	Protein A-ELISA	ABC-ELISA
Cysticercosis	115	0.26 ± 0.156	0.32 ± 0.270	0.31 ± 0.268
Sparganosis	24	0.07 ± 0.081	0.03 ± 0.051	0.03 ± 0.045
T. saginata infection	7	0.09 ± 0.079	0.06 ± 0.085	0.05 ± 0.067
Paragonimiasis	29	0.04 ± 0.020	0.02 ± 0.012	0.02 ± 0.016
Clonorchiasis	28	0.08 ± 0.050	0.02 ± 0.012	0.02 ± 0.008
Other diseases	61	0.08 ± 0.056	0.04 ± 0.030	0.03 ± 0.027
Normal control	16	0.06 ± 0.051	0.02 ± 0.006	0.02 ± 0.004

ABC-ELISA: The general procedures, described in Zymed Lab. (1990), were followed. Briefly, after the chequerboard titrations, 2.5 μg/ml of protein of CF was coated to the microtiter plate. After washing, sera diluted at 1:10,000 in PBS/T (pH 7.4) were reacted for an hour at 36°C. After washing, 1:100,000 diluted biotinylated goat anti-human IgG (heavy- and light-chain specific, Zymed, U.S.A.) were reacted for an hour at 36°C. After washing, peroxidase-conjugated streptavidin, diluted at 1:40,000 in PBS/T (pH 7.4) was reacted for an hour. The reactions were colored by 1:2,500 diluted ABTS in citrate buffer (0.1 M, pH 4.2) containing 0.03% H_2O_2 for 15 minutes. The reactions were terminated by adding 2 mM NaN_3. Abs. was read at 415 nm. The tests were also standardized using positive and negative reference sera.

RESULTS

1. Antibody levels as measured by 3 techniques of ELISA

As shown in Table 2, a total of 115 neurocysticercosis sera was subjected to specific antibody (IgG) measurement by 3 different techniques of micro-ELISA, Protein-A ELISA and ABC-ELISA. Mean abs. and their standard deviations were 0.26 ± 0.156, 0.32

± 0.270 and 0.31 ± 0.268, respectively.

Mean and standard deviation of abs. in 24 patients sera of sparganosis were 0.07 ± 0. 081 by micro-ELISA, 0.03 ± 0.051 by Protein A-ELISA and 0.03 ± 0.045 by ABC-ELISA. Those in 7 *T. saginata* infections, 29 paragonimiasis, 21 clonorchiasis showed lower abs. by Protein A-ELISA and by ABC-ELISA than those by micro-ELISA. Those in 61 other diseases and 16 normal control sera showed similar patterns to other parasitic diseases.

2. Comparison of specific antibody levels between 3 ELISA techniques

Antibody levels (abs.), observed by 3 different ELISA techniques, were compared each other in 115 cysticercosis sera. Fig. 1 showed the relations of individual abs. between micro-ELISA and Protein A-ELISA. Regression equation was $Y = 1.37X - 0.01$ ($r = 0.83$). Cases in negative ranges of antibody levels were either surgically confirmed cysticercosis infected with a few worms or cases in whom CSF only revealed positive antibody reactions. Fig. 2 showed the relations of individual abs. between micro-ELISA and ABC-ELISA. The regression equation was $Y = 1.44X - 0.04$ ($r = 0.86$). Regression equation between Protein A-ELISA and ABC-ELISA was $Y = 0.84X + 0.04$ ($r = 0.84$) (not shown in figure).

Figs. 3-4 showed the relations of individual antibody levels (abs.) by 3 ELISA techniques in sera of *T. saginata* infections, sparganosis, paragonimiasis, clonorchiasis, other diseases and normal control. While 15 cases revealed positive antibody reactions in micro-ELISA, only 3 cases exhibited the positive reactions in Protein A-ELISA or ABC-ELISA.

3. Sensitivity and specificity

Based on cut-off abs. reported by Cho *et al.* (1986), the sensitivity and specificity of the antibody test by micro-ELISA were calculated (Table 3). Sensitivity was 81.7% and specificity was 88.5%. When the cut-off abs. in Protein A-ELISA was set abs. 0.18 as in micro-ELISA, sensitivity was 79.1% and specificity was 97.5%. On the other hand, when cut-off abs. in Protein A-ELISA was lowered to abs. 0.10 (mean + 3 standard deviation of abs.), sensitivity increased to 82.6% while specificity decreased to 93.9%. In case of ABC-ELISA, sensitivity was 78.3% and specificity was 95.8% when the cut-off abs. was set at 0.18. When the cut-off abs. were lowered to abs. 0.10, the sensitivity was increased to 86.1% and specificity was lowered to 94.5%. Statistical tests (Z-test) were done for

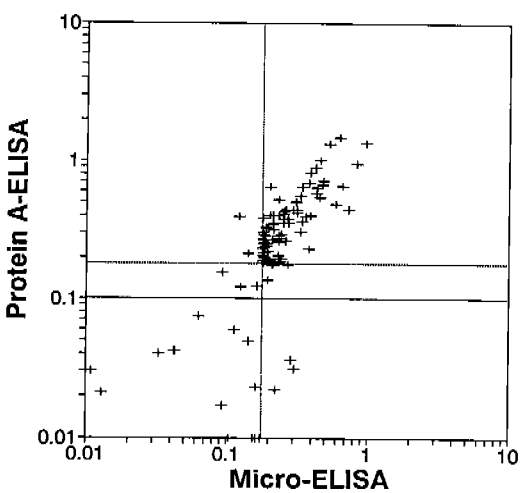

Fig. 1. Relations of the individual antibody levels (abs.) as measured by micro-ELISA and Protein A-ELISA in sera of 115 cysticercosis patients. Horizontal and vertical dotted lines of abs. 0.18 or 0.10 are the positive criteria.

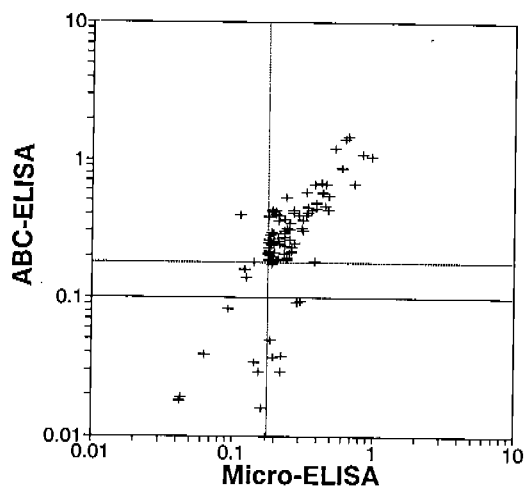

Fig. 2. Relations of the individual antibody levels (abs.) as measured by micro-ELISA and ABC-ELISA in sera of 115 cysticercosis patients. Dotted lines at abs. 0.18 and 0.10 are the same as described in Fig. 1.

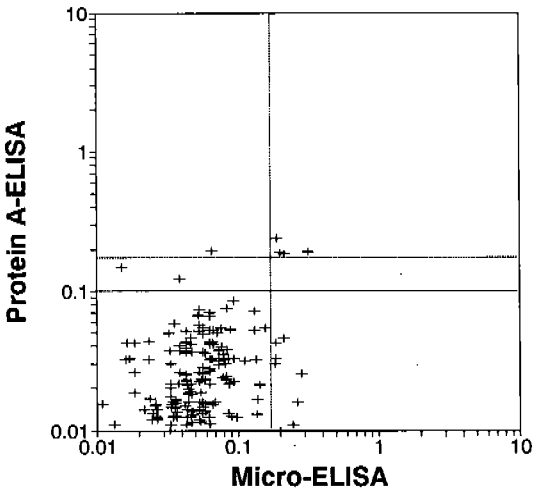

Fig. 3. Distribution and relation of the specific antibody levels (abs.) between micro-ELISA and Protein A-ELISA to *T. solium* metacestode antigen in 165 sera of other diseases and normal controls. Horizontal and vertical lines of abs. 0.18 and 0.10 are the positive criteria.

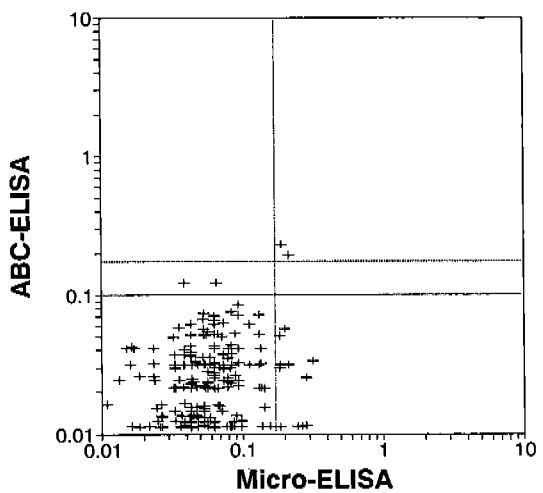

Fig. 4. Distribution and relation of the specific antibody levels (abs.) between micro-ELISA and ABC-ELISA to *T. solium* metacestode antigen in 165 sera of other diseases and normal controls. Lines and symbols are the same as described in Fig. 3.

the significance of the sensitivity and specificity differences between those of micro-ELISA and other 2 ELISA. As shown in Table 3, while the differences in the sensitivities were not significant, those in specificities were statis-tically significant.

Table 3. Sensitivity and specificity of three techqniques of ELISA as calculated in 115 cysticercosis and 165 other diseases and normal control

Techniques	Cut-off abs.	Sensitivity (%)	Specificity (%)
Micro-ELISA	0.18	81.7	88.5
Protein A-ELISA	0.18	79.1*	97.5'
	0.10	82.6**	93.3''
ABC-ELISA	0.18	78.3***	95.8'''
	0.10	86.1****	93.8''''

Z-value with the sensitivity and specificity of micro-ELISA
*0.50 ($p > 0.05$), **0.17 ($p > 0.05$), ***0.64 ($p > 0.05$), ****0.91 ($p > 0.05$)
'3.26 ($p < 0.05$), ''1.52 ($p < 0.05$), '''2.52 ($p < 0.05$), ''''1.71 ($p < 0.05$)

DISCUSSION

Since ELISA technique was proved to be a very useful immunologic tool, it becomes one of the most widely used techniques in measuring antibody, antigen and protein (Voller *et al.*, 1976; McLaren *et al.*, 1979). In addition to standard technique of ELISA using enzyme conjugated secondary antibodies, many modifications of ELISA have been developed utilizing enzyme conjugated materials with non-immunologic binding specificities. For example, applying the specific binding between biotin and avidin, biotinylated secondary antibody and enzyme-conjugated avidin is reacted to form avidin-biotin complex. This ABC-ELISA (Guesdon *et al.*, 1979; Kendall *et al.*, 1983) is now widely applied not only in antigen antibody reactions but also in histochemistry and molecular biology because of its very high sensitivity (Bayer and Wilcheck, 1979).

Another example of ELISA modification is the Protein A-ELISA which utilizes non-immunological binding specificity of staphylococcal Protein A to immunoglobulin G (Kronvall, 1973; Goding, 1978). In Protein A-ELISA, enzyme conjugated Protein A is used instead of enzyme conjugated secondary antibody. This

modification has been known to show higher sensitivity and specificity than standard ELISA. Protein A-ELISA has been applied in diagnosis of parasitic infections (Reed *et al.*, 1990).

In this study, applicability of ABC-ELISA and Protein A-ELISA was evaluated. In specific antibody test of cysticercosis, serum levels of specific antibody were comparable each other when measured by 3 techniques (Figs. 1-4). The correlation coefficients between 3 ELISA were very high (0.83~0.86). ABC-ELISA and Protein A-ELISA showed slightly high values of sensitivity than that of micro-ELISA. However, these were not significant difference. These results indicated that the antibody negative cases of cysticercosis as tested by micro-ELISA was not negative due to low sensitivity of the micro-ELISA itself. Most of the antibody negative cases by micro-ELISA were also negative by more sensitive techniques of ELISA. As Cho *et al.* (1986) reported, antibody levels in different patients of cysticercosis showed the wide variations according to the number of infected worms, duration of infection and different degrees of individual immune responses. Some 20-30% of patients with cysticercosis etiology, therefore, showed comparable serum levels of specific antibody to uninfected normal persons.

In addition to the improved specificity, one significant finding in this study is that samples of serum or CSF can be saved when ABC-ELISA is applied. In micro-ELISA, we dilute serum at 1:100. And 100-200 μl of diluted serum was reacted to pre-coated microplate wells. In ABC-ELISA, however, comparable antibody levels were measured when serum is diluted at 1:10,000. This means that ABC-ELISA is at least 100 times more sensitive in detecting the same reactivity of the specific antibody. This high sensitivity may be very useful in testing CSF levels of the antibody because CSF is very difficult to collect. Once CSF is collected from a patient, physicians try to test all varieties of laboratory examinations such as biochemistry, cell count, cytology, culture *etc.* In our system of micro-ELISA, we use 100-200 μl of undiluted CSF in a single antibody test for cysticercosis. We can now test the comparable quality of the antibody test using only 1-2 μl of CSF if ABC-ELISA is employed.

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=국문초록=

유구낭미충증의 혈청학적 진단을 위한 ABC-ELISA와 Protein A-ELISA의 유용성

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현재 유구낭미충증을 혈청학적으로 진단하는 데에는 낭액항원을 이용한 특이항체검사법으로 micro-ELISA를 널리 이용하고 있다. 이 실험은 효소부착 이차항체를 사용하는 micro-ELISA방법 대신 민감도가 뛰어난 것으로 알려진 ABC-ELISA나 Protein A-ELISA로 바꾸어 사용하면 검사의 민감도를 보다 개선할 수 있는지를 알기 위하여 실시하였다. ABC-ELISA에 의한 항체검사는 낭액 항원 단백질 2.5 μg/ml, 혈청 희석 1:10,000, biotinylated anti-human IgG 1:100,000 희석, peroxidase conjugated streptavidin 1:40,000 희석 및 2,2-azino-di(3-ethylbenzthiazoline) sulfonic acid발색제를 사용하는 조건으로 실시하였고 415 nm에서 흡광도를 측정하였다. Protein A-ELISA는 항원단백질 2.5 μg/ml, 혈청은 1:200 희석, HRP-Protein A 1:20,000 희석 및 ABTS 발색제를 사용하는 조건으로 실시하고 415 nm에서 흡광도를 측정하였다. 유구낭미충증 환자 115명의 혈청을 검사한 바 민감도는 micro-ELISA 81.7%, Protein A-ELISA 82.6%, ABC-ELISA 86.1%이었다. 다른 기생충성 질환자, 비기생충성 질환자 및 대조군 등 165명 혈청에서 특이도는 각각 88.5%, 93.3%, 93.8%이었다. 세 가지 ELISA방법에 의한 항체가 사이에는 상관계수 0.84~0.86의 높은 상관관계가 성립하였다. 이상의 결과 ABC-ELISA는 micro-ELISA에 비하여 민감한 혈청학적 방법이고 실제 유구낭미충 특이항체 검사상의 특이도에서도 차이가 있다는 것을 알 수 있었다. 따라서 ABC-ELISA는 유구낭미충증의 혈청학적 진단에서 혈청 및 뇌척수액 등 검체를 적게 사용할 수 있다는 장점이 있다는 것을 알 수 있었다.

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