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Application of micro-ELISA in serodiagnosis of Human paragonimiasis*

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INTRODUCTION

Since the introduction of bithionol in treatment of paragonimiasis(Yokogawa *et al.*, 1961), the prevalence of this disease have decreased remarkably. Tens of thousands of patients were cured by the treatment. And the population of freshwater crabs (*Ericcheir* spp.) which is one of main sources contracting disease in Korea, reduced to lowest level since the ecology in the rice paddy changed because of the wide use of pesticides in cultivating crops. These two factors resulted in a marked reduction of prevalence and incidence of human paragonimiasis in Korea. However, this disease is still remained as one of the major endemic helminthiases in this country.

Those two factors made parasitologists change their concepts on epidemiology of paragonimiasis. First of all, the endemicity could no longer be expressed by the prevalence rate, and should be shown as an incidence rate in a population. Secondly, the intradermal test using VBS antigen of adult *Paragonimus*, showed lower specificity than it was used in 1950's and 1960's (Sadun and Buck, 1960; Kim, 1969), because many of the infected cases turned to be cured, but still showed positive reaction when tested. Thirdly, the significance of the enzootic cycle in the epidemiology of paragonimiasis should be reevaluated because the infection status of intermediate crustacean hosts remained almost unchanged in spite of the reduction in human infection (Kim, 1969).

So far, most of the epidemiological evaluations of human paragonimiasis have been done using both the intradermal test and intermediate host surveys in many local areas (Rim *et al.*, 1975; Ahn *et al.*, 1979; Lee *at al.*, 1981). Using intradermal test, however, we can no longer surmise that the positive reactors are patients as mentioned above.

To overcome this shortcoming and to evaluate correctly the number of active cases in a community, we should apply either the sputum/stool examination or other specific serological methods which discriminate the patient among the positive reactors of intradermal test. The sputum/stool examination in diagnosis of paragonimiasis has been notoriously known to be low sensitivity because of many chronic or ectopic infections (Kim, 1670). In this respect, the specific IgG antibody tests, such as CF test (Chyu and Lee, 1961), IFA test (Choi *et al.*, 1975; Cho and Soh, 1976) or immunodiffusion test(Yogore *et al.*, 1965; Lee and Choi, 1981) are promising ones because of their high sensitivity and high specificity. In addition, as Choi

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et al. (1976) showed, the specific antibody level returned to negative within 6 months after the successful chemotherapy.

As micro-ELISA technique have been proved as the most convenient serological method for the mass screening of many parasitic diseases (Voller *et al.*, 1976; McLaren *et al.*, 1978; Lee *et al.*, 1981), we evaluated this method in its sensitivity and specificity in diagnosis of human paragonimiasis. By this study we hope that the complicated procedures in mass handling of samples as in CF, IFA and immunodiffusion tests, would be overcomed.

MATERIALS AND METHODS

1. Antigen preparation:

A total of 14 adult *Paragonimus westermani* were harvested from a dog at 13 weeks after the metacercarial infection. The metacercariae were collected from naturally infected crayfishes of Kanghwa Gun, Kyunggi Do (Rim *et al.*, 1975).

After repeated washing in saline to remove the attached or ingested host tissue, the worms were homogenized with teflon-coated tissue homogenizer with 2ml of physiological saline in ice bath. The tissue emulsion was added with 20ml of saline and shaked for 2 hours at room temperature, then kept at 4°C for 24 hours. The emulsion was centrifuged at 4°C by 10,000g for 30 minutes. The supernatant was used as antigen throughout this study (protein content as measured by Lowry method, 1.05mg/ml).

2. The micro-ELISA procedure:

The general procedure of the technique of McLaren *et al.* (1978) was followed. After chequerboard titration of antigen, positive and negative reference sera and conjugate for adequte dilutions, the following procedure was used.

Flat-bottom polystyrene microtiter plate (Dynatech-micro-ELISA) well was coated with 200 microliter of *Paragonimus* antigen which was diluted 1:200 with carbonate buffer (pH 9.6) (protein content, 5.3μg/ml) and overnighted at room temperature. After 3 times of washing of the well with saline/Tween 20 solution, the well was incubated with 200μl of 1:100 dilution of test sera in PBS/Tween 20 (pH 7.4) at 36°C for 2 hours. After washing for 3 times again, 200μl of 1:2,000 dilution of conjugate (peroxidase conjugated antihuman IgG(H&L) goat serum, Cappel Lab., USA) was added and incubated at 36°C for 2 hours. After washing, 200μl of of substrate, made of 99ml of distilled water, 1ml of 1% o-phenylenediamine in methanol, and 50μl of 6% H_2O_2 solution, was added to the well. Checking the degree of colouring of the reference serum, from time to time, the reaction was stopped by 20 μl of 8N H_2SO_4, when the positive reference serum showed optical density, 0.45. The O.D. was read using Spectronic 20 at 492nm with micro-cell adapter.

3. Subjected cases:

As shown in Table 1, a total of 95 carefully selected sera samples was tested in this study. Among them 21 were parasitologically proven, egg positive cases of *Paragonimus westermani* in their sputum. They were selected by field surveys at Miro Myon, Samcheog Gun (3 cases), Bugpyeong Myun, Haenam Gyun, (7 cases), Podu Myun, Goheung Gun (9 cases) and Yangdo Myun, Kanghwa Gun (1 case). Another case was selected at Seoul National University Hospital (Group Ⅰ).

Another group of 8 (Group Ⅱ) was egg negative cases who manifested positive intradermal test for VBS antigen of *Paragonimus*. Two cases out of them showed thoracic lesion radiologically. Another case was that of surgically proven old ectopic paragonimiasis who experienced hemo-

ptysis two decades ago.

Seven cases of *Clonorchis* infection (Group Ⅲ) and 9 cases of other parasitic diseases (taeniasis, cysticercosis and filariasis) (Group Ⅳ) were also tested to observe the possible cross reactions with *Paragonimus* antigen.

The control 50 cases (Group Ⅴ) were carefully selected as non-infected ones of *Paragonimus* by the following criteria; (1) negative intradermal test, (2) repeated negative results for *Paragonimus* eggs in sputum for 3 times, (3) no history of contact with infection sources. Among non-infected cases there were patients of hypertension, liver cirrhosis, bacteriologically proven tuberculosis and normal healthy controls.

Table 1. Subjected cases tested by ELISA

Group	Description	No. of cases
Ⅰ	Egg positive cases of paragonimiasis	21
Ⅱ	Positive rectors of intradermal test for *Paragonimus*	8
Ⅲ	Egg positive cases of *Clonorchis*	7
Ⅳ	Taeniasis, cysticercosis, filariasis	9
Ⅴ	Non-infected controls for paragonimiasis	50
	Total	95

RESULTS

The results, as revealed by optical density at 492nm were plotted in Fig. 1, by the test Groups. The results of each test were highly reproducible when the optical density of the positive and negative reference sera was fixed at 0.45 and 0.02 respectively.

In Group I, 21 egg positive cases of *Paragonimus westermani* showed high antibody level except 3 cases. Optical density ranged from 0.01 to 1.5, and the distribution of the cases were; 3 cases under 0.10, 1 case in 0.25～0.30, 9 cases in 0.31～0.60, 4 cases in 0.61～1.0, 4 cases over 1.0.

In Group II, 2 cases showed high antibody

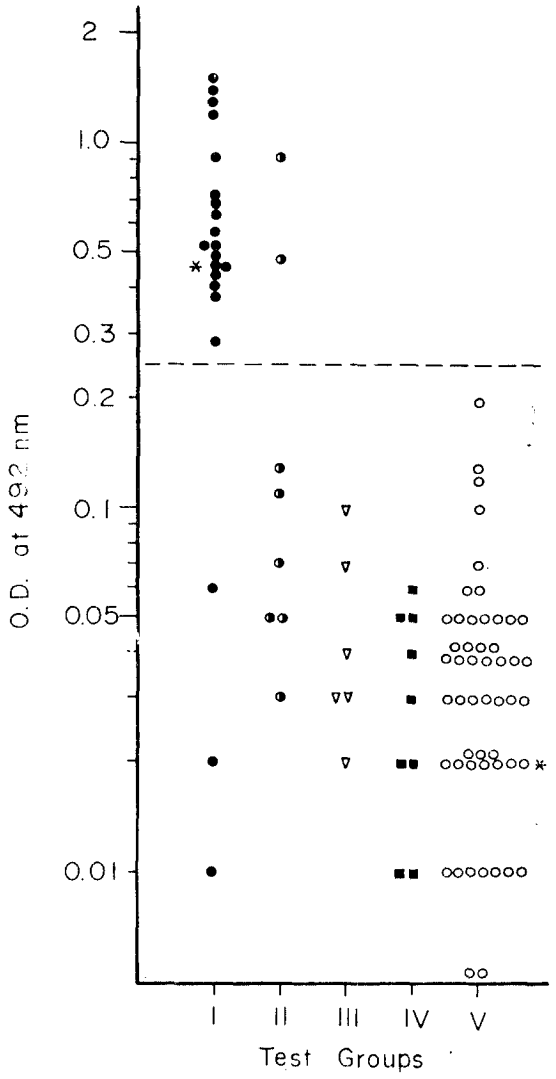

Fig. 1. Result of the enzyme linked immunosorbent assay in subjected cases shown by the Groups. * : Positive and negative reference sera.

level (0.92 and 0.48 in respect); one with thoracic lesion and the other only with positive intradermal reaction. Other 2 cases gave intermediate values, 0.13 and 0.11 respectively; the latter case was old ectopic case. The remaining 4 positive reactors for the intradermal test revealed low IgG antibody level under 0.10.

In Group III (*Clonorchis* infected), the highest antibody level was 0.10 as revealed by optical density. In Group IV (taeniasis, cysticercosis

and filariasis), all cases showed O.D. value under 0.08. In normal healthy controls and other non-infected patients (Group V), the specific antibody level ranged from 0.00 to 0.20(mean±standard deviation; 0.04±0.03).

From these results, we propose the optical density 0.25 as a differential point of positive reaction for presently ill paragonimiasis. By this criterion, the sensitivity of ELISA for the specific IgG antibody was 86% (18 positive reactions out of 21 egg positives). The specificity of this test was 100% in 66 cases of Groups III, IV and V.

DISCUSSION

In this study there was a very interesting case who was very helpful for the setting of the differential criterion of positive reaction. She experienced active hemoptysis two decades ago, and recently received surgical operation because of a mass in pelvic cavity. The pathology of the mass showed no worms at all but old granuloma around the necrotic eggs. This case gave the intermediate antibody level, 0.11 as revealed by optical density. The present authors believe that this kind of old case would not be regarded as positive by means of specific IgG antibody test, even though positive at intradermal test. In addition to this, similar intermediate level of antibody was observed in a *Clonorchis* infected case.

If the differential point of the positive reaction in ELISA is determined by "mean+2 S.D." of the optical density in apparently non-infected cases(Group V in this study), it would be 0.10 (=0.04+0.03×2). Such a low differential point will render many non-infected cases to positive reaction. Considering the present situation of paragonimiasis in Korea, the higher specificity of a diagnostic technique is more desirable than high sensitivity. This is the reason why we set the differential criterion as 0.25, the compromising point, which is below the lowest level of egg positive cases except 3 extraordinarily low ones and upper the control levels.

Three cases out of 21 egg positive paragonimiasis patients showed negative reaction for specific IgG antibody by this criterion. As Kim (1969) observed, even the intradermal test revealed false negative reaction in screening paragonimiasis especially in young age group. In specific IgG antibody test, it is evident that antibody level would not be elevated in all infected cases probably due to immunosuppression or due to antigen-antibody complex. In our study negative reactors were all in age 12 ~15 years.

There observed no cross reaction between *Paragonimus* antigen and other kinds of parasitic infections, especially with *Clonorchis* infected cases. It might be due to high setting of differential criterion. In other respect, it might be secondary to the high dilution of test sera to 1:100. In other kinds of serological methods, such as IFA or immunodiffusion tests, *Clonorchis* infected sera react *Paragonimus* antigen up to serum dilution 1:16(Choi *et al.* 1975; Cho and Soh, 1976).

Through this this observation, specific IgG antibody screening by ELISA for paragonimiasis was found to be highly specific and sensitive, and useful for field survey. However, it is necessary to know something more about the decreasing pattern of antibody level after successful treatment in patients. In addition to this, developing the purified antigens and the analysis of their usefulness might be also desirable.

SUMMARY

To observe whether the specific IgG antibody test using ELISA was useful in diagnosis of

presently ill patients of paragonimiasis, a total of 95 sera were tested. The sera were collected from 21 egg positive cases, 8 from positive reactors of intradermal test, 7 from *Clonorchis* infected, 9 from other parasitic diseases and 50 from apparently non-infected cases.

By the result, the sensitivity of the test was 86% and the specificity was 100%. There were no cross reactions between *Paragonimus* antigen and other parasitic infections.

Specific IgG antibody test by micro-ELISA was concluded to be useful for mass screening of the presently ill paragonimiasis in the field.

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=國文抄錄=

효소면역진단법(ELISA)를 이용한 페디스토마증의 면역진단

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현재 이용하고 있는 페디스토마증의 진단방법의 문제를 개선하기 위하여 이 연구를 실시하였다. 즉 가래 및 대변검사는 그 감도(sensitivity)가 낮고, 피내반응검사는 요즈음 그 특이성(specificity)가 낮아진 상태에 있어 피내반응검사 성적만으로는 현증진단이 매우 어려워진 상태이다. 페디스토마에 대한 특이 IgG항체가 치료 후 6개월 이내에 사라진다는 사실을 이용하여 진단에 이용되고 있다. 그러나 그 방법인 보체결합반응, 형광항체법 및 면역확산법등은 집단검진용으로 쓰이기에는 과정이 복잡하다는 어려움을 갖고 있다.

이상의 여러가지 어려움을 감안하고 효소면역진단법을 이용하였을 때, 특히 집단검사용으로 쓰일 때 그 감도 및 특이 성이 어떠한 것인지를 알아 보고자 하였다.

대상자 혈청은 모두 95명에서 채집하였는 바, 제 1 군(群)인 페디스토마 충란양성자 21명은 삼척군 미로면, 해남군 북평면, 고흥군 포두면, 강화군 양도면 및 서울에서 현지조사를 통해 수집하였고, 페디스토마 피내반응 양성자 8예(제 2 군), 간디스토마 충란양성자 7예(제 3 군), 기타 기생충감염자 9예(제 4 군) 및 페디스토마 피내반응 음성이고 충란검사에서 음성이었던 페디스토마 비감염자 50예등이었다.

검사방법은 Dynatech polystyrene microtiter plate에 페디스토마 항원(3개월된 성충의 생리식염수 추출액 ; 단백함량 1.05mg/ml)을 carbonate buffer(pH 9.6)에 1:200으로 희석사용하고, 환자 혈청은 PBS/Tween 20(pH7.4)에 1:100으로 희석, peroxidase-conjugated antihuman IgG goat serum(Cappel회사제품)은 1:2000으로 각각 희석 반응하였다. 각각의 반응이 끝난 다음에는 Saline/Tween 20용액으로 세척하였다. 끝으로 기질용액(증류수 99 ml+6% H_2O_2 50μl+1% o-phenylenediamine 1ml)을 작용시켰다. 반응착색량을 양성자 표준혈청에서 optical density 0.45일 때 반응을 중지시키고 항체가를 optical density로 표시하였다.

그 결과 충란양성자군과 비감염자군의 O.D.값의 차이가 있는 0.25를 양성반응의 기준값으로 하였을 때 이 반응의 감도는 충란양성자 21예 중 18예가 양성반응이어서 86%이었고, 특이도는 비감염자 66예가 모두 음성이어서 100%이었다.

앞으로 치료받은 환자에서의 반응음성 전환기간에 대한 자료를 보강한다면 페디스토마증의 개별진단 및 집단검진에 유용한 것으로 생각되었다.