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２００１年度日中医学協会共同研究等助成事業報告書

－調査・共同研究に対する助成－

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理　事　長　殿

研究代表者氏名　市　川　秀　隆　㊞
所属機関名　金沢医科大学
部署・役職　医動物学・講師
所　在　地〒 920-0293 石川県河北郡内灘町大学１丁目１番地
電話　076-286-2211　内線　3935

1．研究テーマ　－有鉤嚢虫症 (Cysticercus cellulosae) 診断のための抗原の分離精製並びに、それを用いて中国東北地方の同症感染状況の調査－

2．研 究 期 間　自　2001 年 4 月 1 日 ～ 至 2002 年 3 月 15 日

3．研 究 組 織

日本側研究者氏名　市　川　秀　隆
所属機関　金沢医科大学　職名　講師

中国側研究者氏名　徐　之傑
所属機関　哈爾濱医科大学　職名　教授

4．研究報告書

別紙報告書作成要領に従い、添付の用紙で研究報告書を作成して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

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5．収支決算報告

--有鉤嚢虫症 (Cysticercus cellulosae) 診断のための抗原の分離精製

2001年4月4日交付通知のあった研究課題 並びに、それを用いて中国東北地方の同症感染状況の調査--

についての収支決算を行ないました。領収書コピーを添えて、次のとおり報告します。

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科目区分	金額	備考（用途・内訳）
消耗品	1,000,000	抗ヒト血清 IgG(α) ヤギ HRP 抗ヒト血清 IgG【F(ab′)2】ヤギ HRP 抗ヒト血清 IgM(μ) ヤギ HRP 抗ヒト血清 IgE(ε) ヤギ HRP 抗ブタ全血清ウサギ HRP シルバーステイン SDS-PAGE キット ABTˆS DAB BSA TEMED アクリルアミド ビス 過硫酸アンモニウム フレーム モジュール マイクロピペット P-200 マイクロピペット P-100 マイクロピペット P-10 ダイヤモンドチップ ドラモンドピペットエンド PA-300 エッペンドルフピペット 4780 その他

—日中医学協会助成事業—

—有鉤嚢虫症（Cysticercosis cellulosae）診断のための抗原の分離精製並びに、それを用いて中国東北地方の同症感染状況の調査—

研究者氏名　市川秀隆

日本研究機関　金沢医科大学医動物学

共同研究者　伊藤洋一[*1]・保阪幸男[*1]・小松俊彦[*1]・長瀬啓三[*3]・徐　之傑[*4]

[*1]バイオメディカルサイエンス研究会

[*3]藤田保健衛生大学短期大学医動物学教授

[*4]中国・哈爾濱医科大学寄生虫学教授

ABSTRACT:

Immunoserological study of cysticercosis carried out on sera from patients, healthy Chinese, endemic inhabitant and non-endemic inhabitant, using the enzyme-linked immunosolbent assay (ELISA) and enzyme-linked immunotransfer blot assay (EITB) in Jilin, China.

For the evaluate of suitable antigen, we prepared the 2 materials designated as cyst fluid (CF) and whole cyst (WC) obtained from pig. A total 430 sera, including 134 healthy sera, 86 patients sera, 115 non-endemic inhabitants, 95 endemic inhabitants sera obtained from Changchun in China. In patients (clinically suspected) sera, 82 (95.4%) out of 86 sera were positive. On the other hand, 4 (3.0%) of 134 healthy Chinese sera showed positive. Sixteen (13.9%) out of 115 sera from non-endemic area were positive. On the other hand, 33 (34.7%) of 95 sera of endemic area showed positive. In comparison with ELISA value among sera from healthy Chinese, non-endemic and endemic area inhabitants, clearly significant difference were observed.

Key Words : Cysticercosis, seroimmunodiagnosis, ELISA, Immunoblot

INTRODUCTION:

Cysticercosis, caused by larval stage of *Taenia solium*, is a serious problem to human health. The disease is endemic in a few countries of Latin America, South-east Asia, and Central Africa and is becoming increasingly prevalent in the United States and Europe. The larvae migrate into central nervous system (CNS) of human and were induced reveal severe neurological disorders. Diagnosis study evaluting the cysticercosis lesions with nuclear magnetic resonance imaging (MRI) and comuputed tomography (CT) is proposed. Recently, Plancerte et. al. (1994) demonstrated that enzyme linked immunosolvent assay (ELISA) and immunoblot assay was a good diagnostic tool for cysticercosis. But specific diagnosis of this disease is difficult until now.

Seroepidemiological survey has been carried out in several endemic countries by using with ELISA. In Central America, especially in Mexico, there are many reports of serodiagnostic survey of cysticercosis. However, as far as we

know, there are few from China, despite its endemicity.

The prevalence of cysticercosis was investigated among non-endemic and endemic area in Jilin of China. The availability of ELISA with enough sensitivity and especificity to be applied in the field setting, permitted to perform a series of populational studies aimed to know the epidemiological characteristic of cysticercosis in endemic populations in China.

MATERIALS AND METHODS:

Preparation of antigens

The cysticerci were obtained from infected pigs and separated the 2 parts of cyst fluid (CF) and whole cyst (WC). Each material was homogenized in 3 volumes of 0.85% NaCl solution using a Teflon-glass homogenizer in ice. The homogenates were centrifuged at 15,000 G for 30 min, and the supernatants were used as antigen.

Protein concentrations were determined by the Bio-Rad protein assay kit (Bio-Rad Lab., Richmond, CA) using a bovine serum albumin as standard.

Sera

Eighty-six sera from clinically suspected case of cysticercosis and 134 sera, from healthy individuals without history of neurological disorders were obtained in Changchun, China. Ninety-five sera from inhabitants in endemic area of Shaangyáng and 115 sera from non-endemic area of Nóngan, China were also obtained. Sera from 134 healthy Chinese were used as control.

ELISA studies

The protocol for ELISA method followed Engvall and Peter (1971) and Weekmen and Schcrus (1971). Briefly,. microtiter plates (NUNC, Intermed, Denmark) were coated with 100μl (10μg/ml protein) of antigen solution (0.5M carbonate-bicarbonated buffer, pH 9.6) and incubated for one hour at 38°C and left overnight at 4°C. The excess antigen was washed away using 0.05% Tween-20 in PBS (PBS/T). Blocking was done with 100μl of PBS/T containing 1% bovine serum albumin (BSA) for one hour at 38°C. After the washing, 100μl of diluted serum samples was added to wells. The last well of each plate received serum-free PBS/T and served as negative control or blank well. The plate was incubated for one hour at 37°C and was washed several times with PBS/T. Peroxidase-labeled goat anti-human immunoglobuline G Fc-Fragment (Organon Tekhica N.V.-Cappel Productes, USA) at dilution 1:500 in PBS/T was added to all wells (100μl per well) and the plate were incubated for one hour. After washing, we added the 100μl substrate, 0.03% 2,2'-azino-bis(3-ethyl-benzthiazolin-6-sulfonic acid) in 0.05M citric acid and 0.01M sodium phosphate buffer containing 0.003% H_2O_2, to each well. The enzyme-substrate reaction was allowed to occur at room temperature for 60 minutes. The reaction was stopped by adding 50μl of 1.25% sodium fluoride to each well. The optical density (OD) of each well was measured at 405-595 nm against the blank using Model 3550 microplate reader (Bio-Rad Laboratories, USA).

SDS-PAGE and Immunoblotting

SDS-PAGE was performed according to the procedure of Laemmli (1970) in 12.5% polyacrylamid gels. In some experiments, disulfide bonds were reduced with 5% ß-mercaptoethanol. The protocol of electrophoretic transfer onto nitrocellulose followed Burnet (1981). The samples were separated with SDS-PAGE under reducing conditions, then electrotransferred to a nitrocellulose membrane (GVHP 304FO, Nihon Millipore Kogyo Co., Yonezawa, Japan). Some membrane strips were stained with Indian ink for protein profiles (Hancock and Tsang, 1983). The remaining strips were immunostained with peroxidase conjugated anti-human IgG antibody as follows; the strips were washed, incubated in bloking solution (1% BSA/T), then reacted with sample sera diluted 1/100 in PBS/T. After incubation in peroxidase-conjugated anti-human antibody solution diluted 1/500 in PBS/T, the nitrocellulose strips were incubated with the substrate, 0.7 mM 3,3'-diaminobenzidine tetrahydrochloride, 0.01% H_2O_2, 50mM Tris-HCl buffer, pH 7.2. The reaction was stopped by rinsing the strips with tap water.

A molecular standard (M 4038, Shigma Chem. Co., St Louis, USA) was run with each gel.

Statistics

Statistical differences was analyzed by the t-test. A p-value of less than 0.01 was considered to be significant.

RESULTS AND DISCUSSION:

The diagnosis of CNS cysticercosis is often difficult. Palpation for subcutaneous nodules and roentgenograms for calcified cysts in muscles occasionally aid the diagnosis. In the past, indirect hemagglutination and complement fixation tests has been utilized, but in our experience, neither has been sufficiently reliable to permit the desired degree of confidence in serological diagnosis. Recently, CT scans have been employed with considerable success and are especially helpful in determining the need for surgery .

We carried out to know the optimum concentration of serum by ELISA test, which reacted with 1μl of WC antigen from Guatemalan cysticerci. The results showed 1024 times or more dilution of healthy Chinese sera has only a few background response (Fig. 1). As a result of these data, 1000 times dilution of sera were used to detect antibodies by ELISA.

Fug.2 showed the ELISA using with WC and CF antigens in sera from healthy, patient, non-endemic and endemic area inhabitants. Scatter plots showed the result of ELISA value measured in using WC and CF antigens. A few strong reactions with WC antigen than CF ($y=0.287x + 0.032$) were observed on healthy Chinese sera (Fig. 2-A). But patient, non-endemic inhabitant and endemic inhabitant sera showed almost equivalent reaction with WC and CF as $y=0.924x - 0.029$, $y= 0.854x +0.049$, $y=0.699x + 0.048$, respectively.

The mean OD values with SD at a dilution of 1:1000 in 134 Chinese sera were 0.108±0.063 and 0.064±0.027, respectively (Table 1). We were defined means±3SD as the cut-off value between positive sera and negative. According to cut-off value of both antigens, 82 (95.4%) out of 86 sera from clinically suspected were positive, but 4 (3.0%) out of 134 healthy sera showed also positive (Table 2). In endemic area, 33 (34.7%) out of 95 sera were positive, but 16 (13.9%) out of 115 sera from non-endemic area showed positive (Table 2).

ELISA value between WC and CF antigens were shown Fig. 2, and sera from patient, non-endemic and endemic

inhabitants were almost same value. On the other hand, the ELISA value of WC antigen were higher than CF antigen in healthy Chinese sera (Fig. 2-A).

In order to characterize WC and CF antigen fraction, with 84 sera (22 were healthy persons, 22 were patients, 18 were non-endemic area persons and 22 were endemic area persons) showing a high OD value against WC and CF antigens fraction by ELISA were examined by immunoblot assay (Fig. 3,4,5,6).

In patient sera, 22 of them reacted with 15.5, 17.5, 18.5, 20, 22, 26.5, 27, 28, 32, 35, 37, 40, 48, 56, 62, 82, 97, and 115 kDa MW on WC antigen and 35 of them (Fig. 4) were reacted with 15.5, 17.5, 18.5 22, 26.5, 27, 28 and 97 kDa MW on CF antigen, respectively. But, some of them showed nothing reaction whether it showed higher OD value with two antigens by ELISA.

Similarly, in non-endemic area, which sera showed positive reaction on the ELISA were reacted with 15.5, 17.5, 18.5, 20.5, 22.5, 26.5, 27, 28, 32, 35, 37, 40, 48 56, 62. 82 and 97kDa MW on WC antigen and were reacted with 27 and 39 kDa MW on CF antigen (Fig. 5), respectively.

In endemic area, which sera showed positive reaction on the ELISA were reacted with 15.5, 17.5, 18.5, 20.5, 22.5, 26.5, 27, 28, 35, 37, 40, 48, 56, 62, 82, 97 and 115 kDa MW on WC antigen and were reacted with 35, 37, 40, 80 and 97kDa MW on CF antigen (Fig. 6), respectively.

Guerra et al. (1982) described antigen B to be composed of 2 protein subunits with molecular weights of 105 and 95 kDa. Analysis of the antigenic profiles obtained by us reveals polypeptides with the similar molecular weight (95 and 115kDas). We found that the smaller of these antigens (97 kDa) reacted with IgG. In addition, it is also clear from our results that antigens with molecular weights of 62,65, 48, 40, 37, 35, 28, 27, 26.5 kDa were immunodominant.

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Table 1.
OD value with serological test by ELISA using WC and CF antigens of cysticercus cellulosae

Serum		Normal	Non-endemic area	Endemic area	Patient
No. of person		134	115	95	86
ELISA OD value (means7SD)	WC	0.10870.063	0.14670.165	0.20570.232	0.62170.191
	CF	0.06470.027	0.11470.184	0.22470.288	0.70470.197

WC: whole cyst
CF: cyst fluid

Taible 2.
Number of positive sera with ELISA using WC and CF antigens of cysticercus cellulosae

Serum		Normal		Non-endemic area		Endemic area		Patient	
No. of person		134		115		95		86	
No. of positive person* (%)	WC	2 (1.5%)	4 (3.0%)	12 (10.4%)	16 (13.9%)	25 (26.3%)	33 (34.7%)	80 (93.0%)	82 (95.4%)
	CF	2 (1.5%)		13 (11.3%)		32 (33.7%)		82 (95.4%)	

* Positive limit by control data (_means _3SD) of Chinese on WC and CF antigens
WC: whole cyst
CF: cyst fluid

Fig. 1. Optimum concentration of normal chinese person sera which measured absorbance of ELISA using on WC antigen

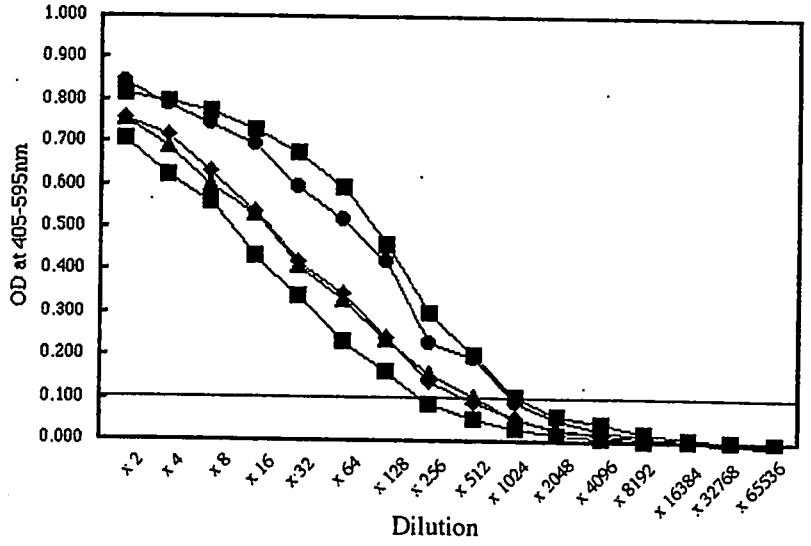

Fig 2

Correlation of ELISA value between WC and CF antigens in hearthy, patient, Non-endemic area and endemic area sera

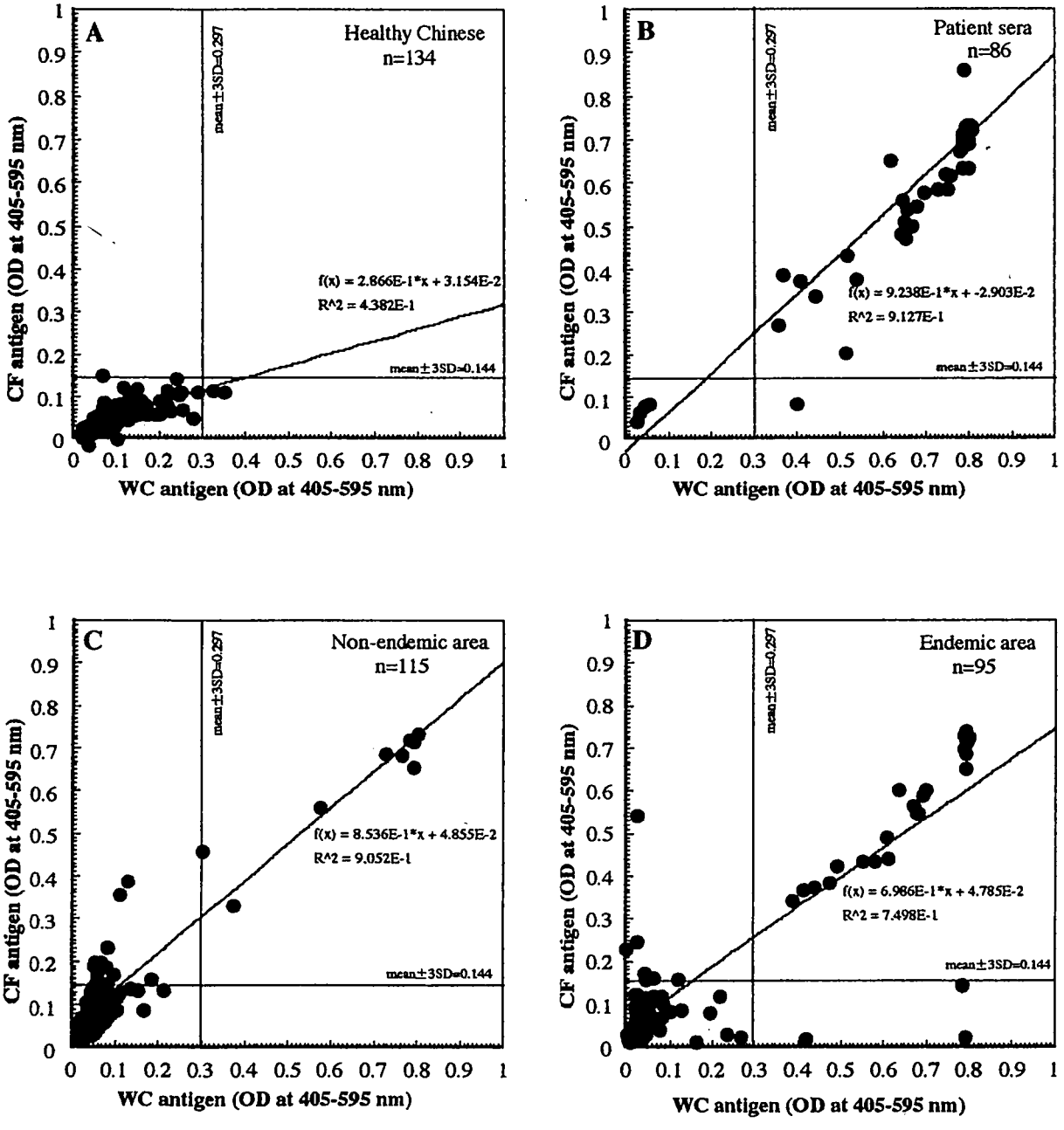

Fig. 3 Immunoblot analysis of whole cyst and cyst fluid antigens of cysticercus cellulosa with healthy Chinese sera

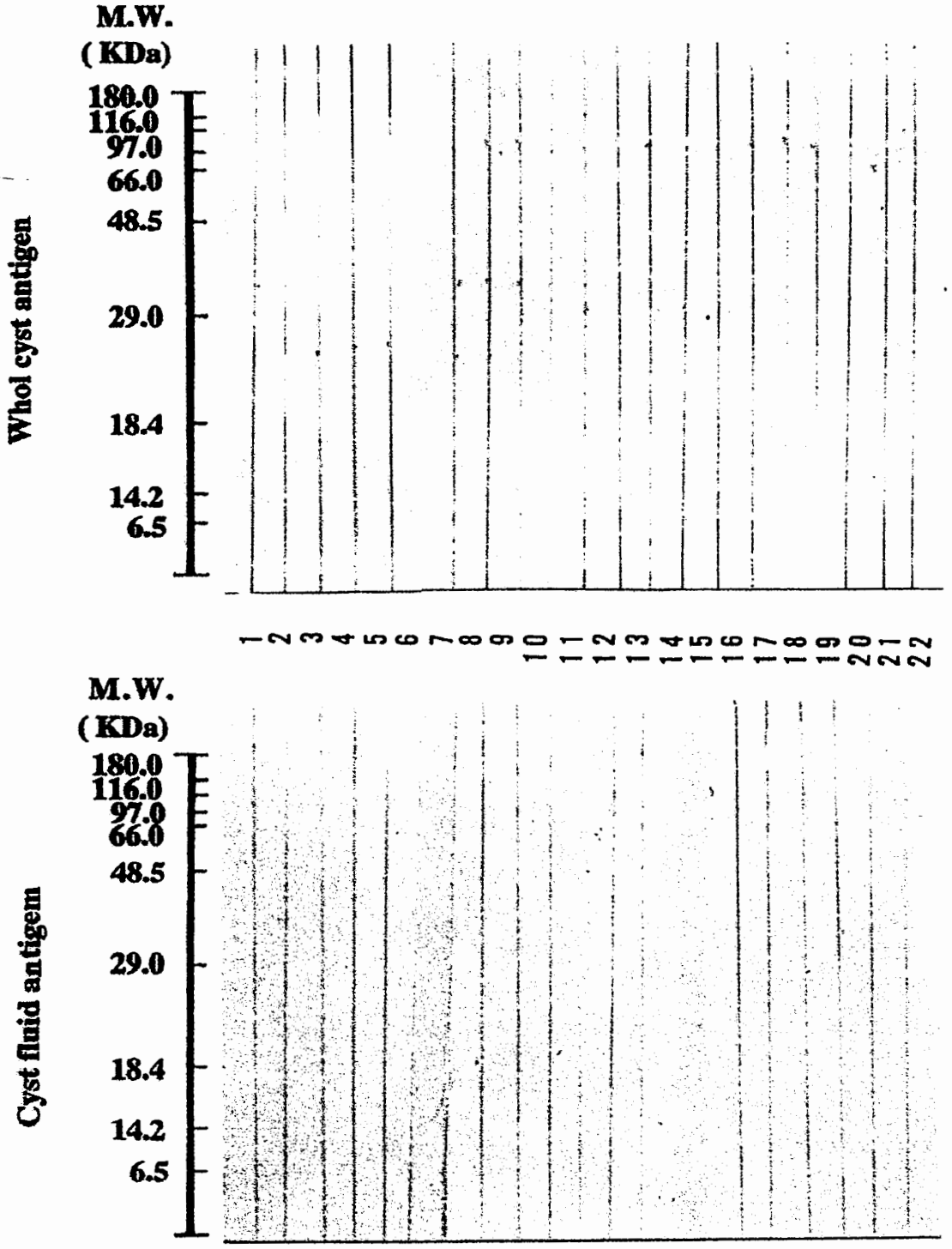

Fig. 4 Immunoblot analysis of whole cyst and cyst fluid antigens of cysticercus celllosa with patient sera

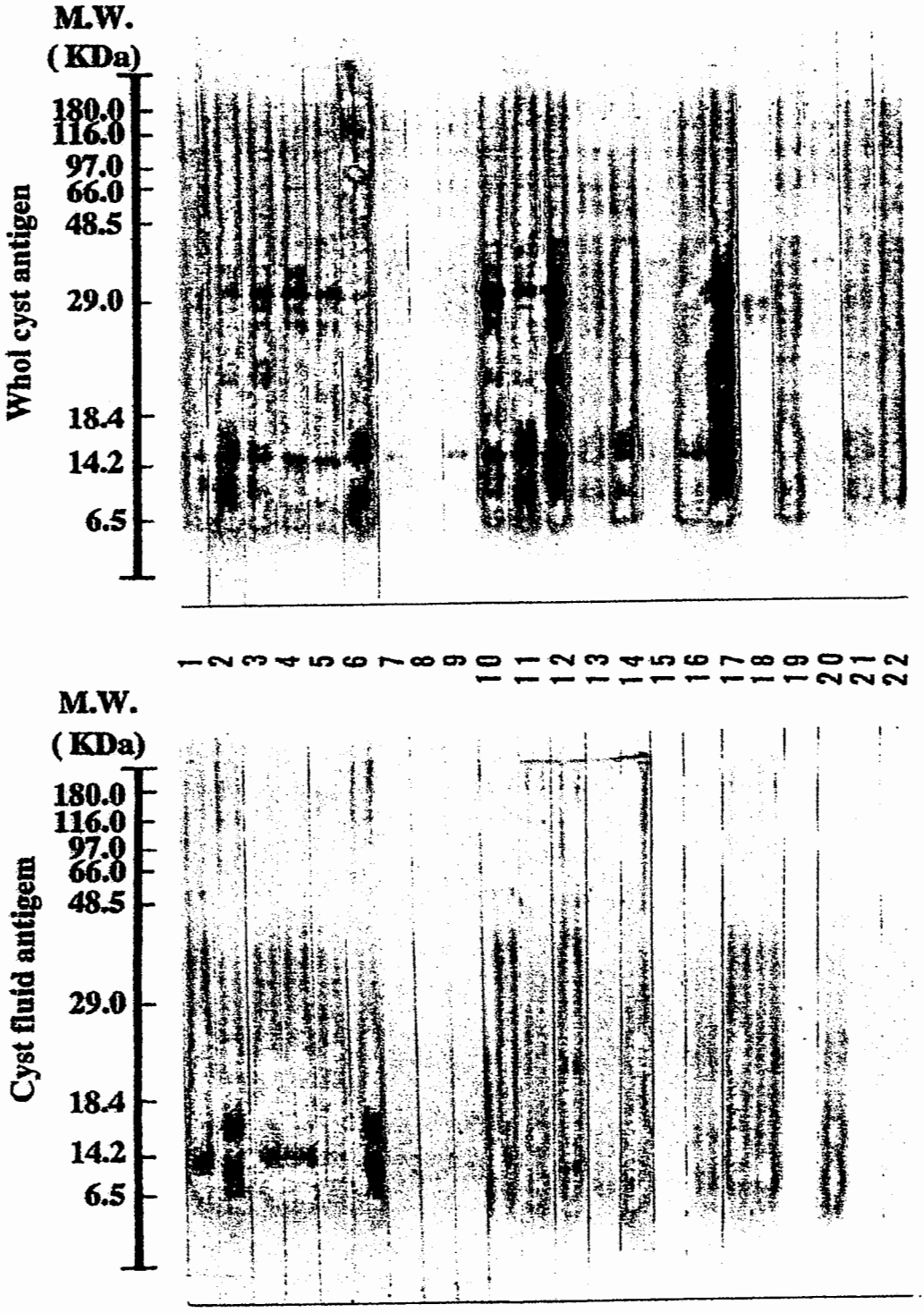

Fig. 5 **Immunoblot analysis of whole cyst and cyst fluid antigens of cysticercus cellulosa with non-endemic inhabitant sera**

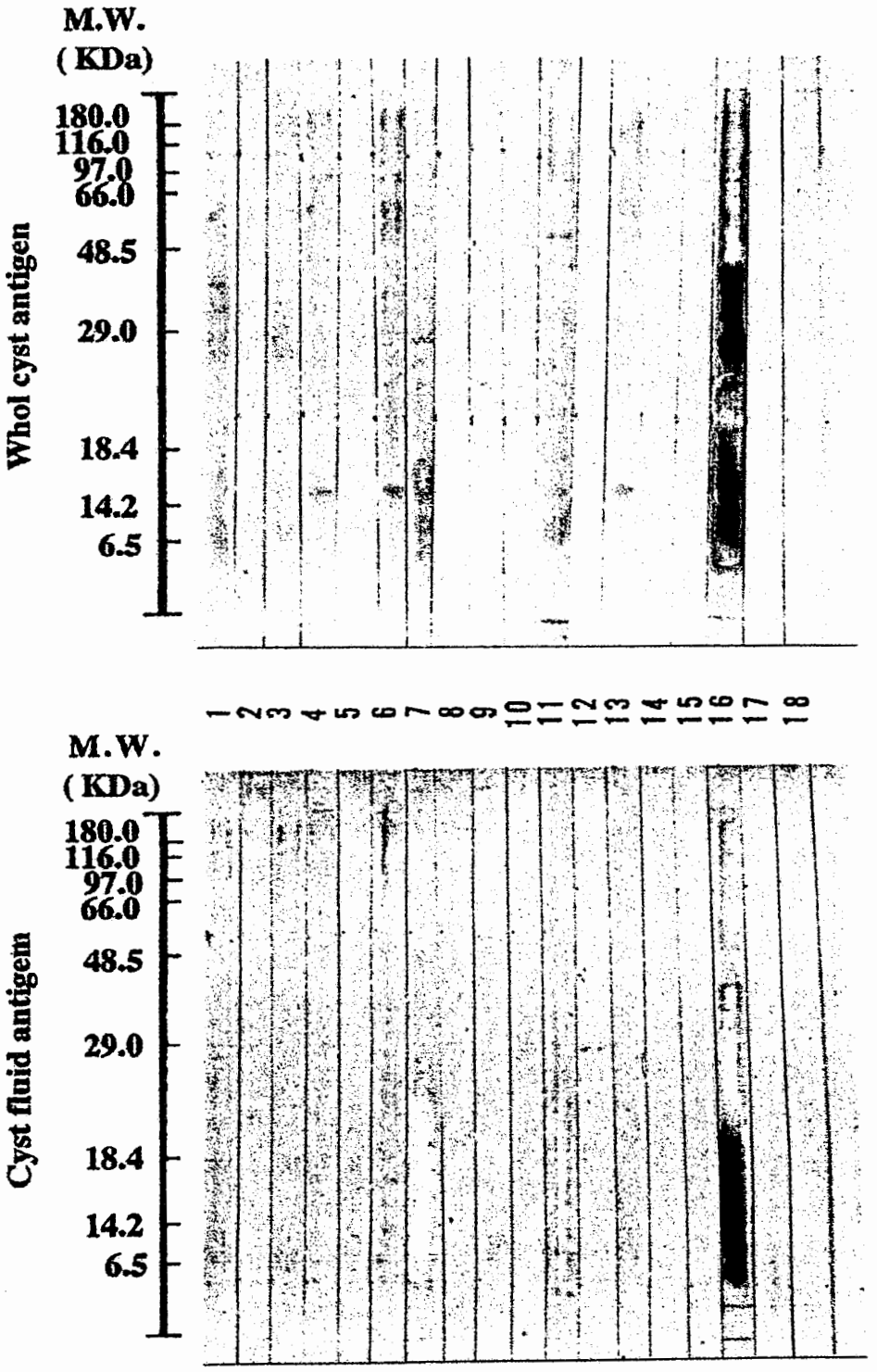

Fig. 6 Immunoblot analysis of whole cyst and cyst fluid antigens of cysticercus cellulosa with endemic inhabitant sera