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A Survey of Antibody to *Toxoplasma gondii* among Patients of a Hospital in Hyogo Prefecture, Japan, by Enzyme-Linked Immunosorbent Assay

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Introduction

Toxoplasmosis is an severe disease caused by a protozoan parasite, *Toxoplasma gondii*. Especially its congenital infections in infants and acute infections in immunosuppressed patients are generally considered to be important in diagnostic and therapeutic medicine. This parasite is ubiquitously distributed in the world and its prevalence in humans is observed in most countries. In Japan, Kobayashi *et al.* (1974) reported 25.3 % of the prevalence rate in pregnant women 18 to 45 years old, and another antibody surveillance performed in Tokyo (Kobayashi, 1977) indicated that the prevalence rate was almost equal to two thirds of the age value in general inhabitants, as assessed by the dye test or the indirect hemagglutination test. Recently, Ise *et al.* (1981) have reported 21.3% prevalence in pregnant women with the latex agglutination test.

The present study was designed to reveal the current situation of *Toxoplasma* prevalence among inhabitants in or near Hyogo Prefecture, by the newly-established immunodiagnostic method, enzyme-linked immunosorbent assay (ELISA). This technique provided us a rapid and precise quantification of antibody levels in sera (Konishi and Takahashi, 1983). This paper deals with the survey of *Toxoplasma* antibody among patients of our Hospital using the ELISA technique. Comparison was made in qualitative and quantitative results between male and female, and between age groups.

Tachyzoites for preparation of ELISA antigen have been collected by the differential centrifugation method in our previous studies. In this study, the filtration method with a polycarbonate membrane (Handman and Remington, 1980) was introduced, which resulted in high recovery with low mouse cell contamination. Comparison of these two methods is also described in this paper.

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Materials and Methods

Antigen

Antigen was prepared from tachyzoites of the RH strain of *T. gondii* harvested from mice that had been infected 3 days before. The organisms were collected by two ways: one is differential centrifugation as previously described (Konishi and Takahashi, 1983) and the other is filtration through a polycarbonate membrane (Nuclepore, Corp.) with a porosity of 3 μm (Handman and Remington, 1980). The parasites in the filtrate were then washed and suspended in phosphate-buffered saline (PBS; pH 7.4), and disrupted by sonication at 28 kHz five times for 1 min each. After centrifugation at 10,000$\times g$ for 1 h, the supernatant was stored at -20°C until used as *Toxoplasma* antigen.

Sera

Sera taken routinely from patients were supplied from the Central Laboratory of our Hospital. A total of 1,331 sera randomly

sampled out of 5,100 sera provided from July, 1981 to January, 1982 were used. The ages of patients examined ranged from one to 93 years and samples from less than one year old were removed, because antibodies transferred from mothers were maintained for about six months. The ages were grouped with an increment of 10 years ; 1-9, 10-19, 20-29, 30-39, 40-49, 50-59, 60-69, 70-79 and 80-93 years.

ELISA system

The ELISA test with a magnetic processing system was performed essentially as previously described (Konishi and Takahashi, 1983) unless otherwise specified. In brief, solid-phase polycarbonate-coated iron beads were sensitized with 15 μg/ml of *Toxoplasma* antigen. After three washes with PBS containing 0.05 % Tween 20, the beads were reacted with 100 μl of 100-fold diluted test sera in microplate wells at 37°C for 1 h. They were then processed with alkaline phosphatase-conjugated antihuman IgG (prepared as described by Engvall and Perlmann, 1972) at 37°C for 1 h, followed by the reaction with the substrate, 0.1 % *p*-nitrophenyl phosphate, at 37°C for 20 min. The absorbance of the reaction mixture was measured at 410 nm by Microelisa Minireader MR 590 (Dynatech Laboratories). Two beads were used per test sample and the absorbance values obtained by two beads were averaged and adjusted with the value for the positive control as 1.0.

For qualitative analysis, sera were diagnosed as positive, doubtful, and negative, when the value was more than 0.357, between 0.266 and 0.357, and less than 0.266, respectively.

Results

Comparison of antigens prepared by differential centrifugation and filtration methods

The filtration method was about three times as rapid as the differential centrifugation : thus, physiological saline was enough for suspending organisms in the former, while Eagle's minimum essential medium was needed in the latter. Recovery rate was higher than 80 % in filtration and less than 30 % in differential centrifugation and mouse cell contamination was less than 0.1 % in filtration and more than 10 % in differential centrifugation.

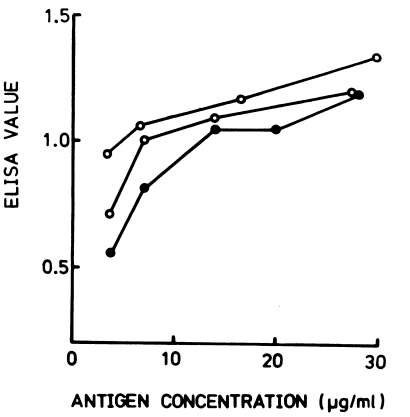

Fig. 1 Potency of *Toxoplasma* antigen prepared by filtration and differential centrifugation methods in the ELISA system for quantification of antibodies in sera. Solid phase iron beads were sensitized with antigens at various protein concentrations as measured by the method of Lowry *et al.*(1951). A single positive sera was used for the first antigen-antibody reaction. The other reaction was performed as described in Materials and Methods. Two lots of antigen prepared by filtration on different days (○) and one preparation by differential centrifugation (●) were compared in ELISA value as an indicator of antigenic potency.

Two antigen preparations provided almost the same antigenic potencies at higher than 15 μg/ml of antigen concentrations for solid phase sensitization. Dose response curve, however, leveled off at different antigen concentrations in preparations by filtration (ca. 7 μg/ml) and differential centrifugation methods (ca. 15 μg/ml). The difference may be related to the antigen purity due to mouse cell contamiation at the *Toxoplasma* collecting process.

Qualitative result of ELISA test for Toxoplasma antibody

Diagnosis of 1,331 patient sera by the ELISA testing system is summarized in Table 1. Random sampling of the sera resulted in a greater number of female samples (863 sera) than male (468 sera). The positive ratio

Table 1 Diagnostic result of 1,331 patient sera for *Toxoplasma* antibody as tested by ELISA system

Age (years)	Male				Female				Total			
	Pos.	Doubt.	Neg.	Total	Pos.	Doubt.	Neg.	Total	Pos.	Doubt.	Neg.	Total
1— 9	2 * (5.6)	0 (0.0)	34 (94.4)	36	2 (5.4)	0 (0.0)	35 (94.6)	37	4 (5.5)	0 (0.0)	69 (94.5)	73
10—19	1 (1.6)	1 (1.6)	59 (96.8)	61	9 (6.3)	2 (1.4)	133 (92.3)	144	10 (4.9)	3 (1.5)	192 (93.6)	205
20—29	3 (8.1)	2 (5.4)	32 (86.5)	37	4 (3.0)	4 (3.0)	124 (94.0)	132	7 (4.1)	6 (3.6)	156 (92.3)	169
30—39	12 (19.7)	2 (3.3)	47 (77.0)	61	16 (10.7)	5 (3.4)	128 (85.9)	149	28 (13.3)	7 (3.3)	175 (83.4)	210
40—49	13 (26.0)	3 (6.0)	34 (68.0)	50	22 (17.7)	4 (3.2)	98 (79.1)	124	35 (20.1)	7 (4.0)	132 (75.9)	174
50—59	24 (26.7)	6 (6.7)	60 (66.6)	90	19 (16.2)	5 (4.3)	93 (79.5)	117	43 (20.8)	11 (5.3)	153 (73.9)	207
60—69	22 (25.0)	7 (8.0)	59 (67.0)	88	27 (26.2)	2 (1.9)	74 (71.9)	103	49 (25.7)	9 (4.7)	133 (69.6)	191
70—79	14 (36.8)	3 (7.9)	21 (55.3)	38	10 (25.0)	2 (5.0)	28 (70.0)	40	24 (30.8)	5 (6.4)	49 (62.8)	78
80—93	4 (57.1)	0 (0.0)	3 (42.9)	7	5 (29.4)	1 (5.9)	11 (64.7)	17	9 (37.5)	1 (4.2)	14 (58.3)	24
Total	95 (20.3)	24 (5.1)	349 (74.6)	468	114 (13.2)	25 (2.9)	724 (83.9)	863	209 (15.7)	49 (3.7)	1073 (80.6)	1,331

* Number in parenthesis indicates percentage frequency.

was 15.7 % in total and it tended to increase with age. Similar ratios were obtained for age groups of 1-9, 10-19 and 20-29 years and it rose about three times at 30-39 years and then increased gradually, reaching 37.5 % at 80-93 years. As for sex difference, total prevalence in male (20.3 %) was much greater than that in female (13.2 %); the difference was statistically significant ($p<0.001$). When comparison was made in each age group, all except 10-19 and 60-69 years indicated higher values in male. But in these cases, differences were not so great as to be significant ($p>0.05$).

Quantitative result of ELISA test for Toxoplasma antibody

Distribution of ELISA values was compared between male and female (Fig. 2). The negative group consisting of sera with ELISA values of 0.0 to 0.266 had a clear peak at 0.05-0.10 in both distributions. In contrast, the distribution pattern of positive group (ELISA values of more than 0.357) was not observed as such an obvious pattern; the

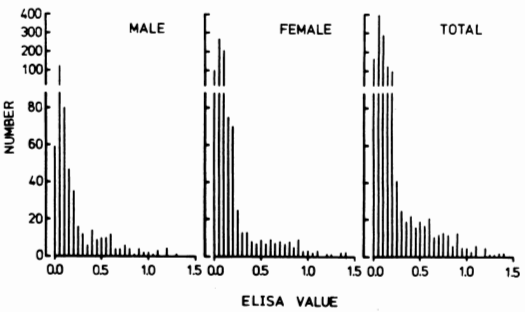

Fig. 2 Frequency distributions of ELISA values for 468 male, 863 female and totaling 1,331 sera of patients in our Hospital.

frequency gradually decreased with increase of ELISA value. In statistical analysis (Table 2), the mean ELISA values for negative and positive groups were calculated to be, respectively, 0.1100 and 0.6634 in male, and 0.1087 and 0.7092 in female. Two-sample *t*-test (Campbell, 1967) indicated no significant difference between male and female both in positive and negative groups ($p>0.05$).

Frequency distribution was studied to know

Table 2 Statistical analysis of ELISA values for *Toxoplasma* antibody each in positive and negative groups for estimating the difference between male and female

Population	Negative group			Positive group		
	No.	Mean	SD*	No.	Mean	SD
Male	349	0.1100	0.0638	95	0.6634	0.2355
Female	724	0.1087	0.0595	114	0.7092	0.2572
Total	1,073	0.1091	0.0609	209	0.6884	0.2480

* Standard Deviation

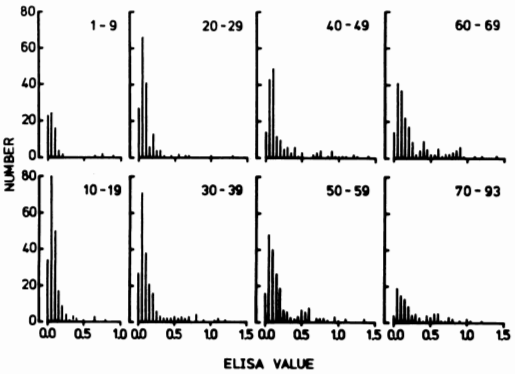

Fig. 3 Frequency distributions of ELISA values for patient sera of 8 age groups obtained in our hospital.

age differences in this population (Fig. 3). The age group of 80–93 years was incorporated into that of 70–79 years, because of its small sample number. The patterns were essentially similar to those shown in Fig. 2, in that negative groups had clear peaks in contrast to positive groups. However, changes with age were observed in population sizes of positive and negative groups according to an increase of the prevalence rate (Table 1). Moreover, the statistical analysis (Table 3) indicates a gradual increase of mean ELISA value in the negative group. Difference between age groups of 1–9 and 70–93 years was significant ($p<0.001$) in the two-sample t-test. As for the positive group, mean values were not significantly different ($p>0.05$), except for an age group of 10–19 years, which was smaller in mean than those of 1–9 and 40–49 years with the level of significance of $p<0.01$ and $p<0.05$, respectively.

Discussion

Enzyme-linked immunosorbent assay system was adopted in the survey of *Toxoplasma* antibody in Hyogo Prefecture, Japan. Although the dye, indirect hemagglutination and latex agglutination tests were used in lit-

Table 3 Statistical analysis of ELISA values for *Toxoplasma* antibody each in positive and negative groups for estimating the difference between age groups

Age (years)	Negative group			Positive group		
	No.	Mean	SD*	No.	Mean	SD
1— 9	69	0.0801	0.0516	4	0.7875	0.1031
10—19	192	0.0971	0.0546	10	0.5350	0.1647
20—29	156	0.0997	0.0594	7	0.7679	0.3020
30—39	175	0.1061	0.0608	28	0.6946	0.2354
40—49	132	0.1148	0.0588	35	0.7507	0.2966
50—59	153	0.1230	0.0631	43	0.6890	0.2460
60—69	133	0.1224	0.0623	49	0.6730	0.2494
70—93	63	0.1353	0.0661	33	0.6568	0.2135

* Standard Deviation

eratures reported so far (reviewed by Kobayashi, 1977; Ise *et al.*, 1981), the ELISA is best suited for mass-survey because of its simplicity, rapidity and economy, in addition to reliability as testing system for *Toxoplasma* antibody (Konishi and Takahashi, 1983). About 200 serum samples were assayed by one person per day and an antigen preparation obtained from one infected mouse served the testing of about 1,000 samples.

Antigen preparation is one of the problem in performing ELISA in laboratories because the antigen is not commercially available in general except the "solid-phase" antigen as in kits. High recovery with high purity is required when *Toxoplasma* organisms are collected from mouse peritoneal exudates. The use of a polycarbonate membrane filter with 3 μm porosity was first reported by Handman and Remington (1980) and they mentioned the viability of tachyzoites of greater than 95 % with host cell contamination of less than 0.1 %. In this paper, the filtration method with a polycarbonate membrane was compared with the differential centrifugation that we have previously used, and it was found that the former was superior to the latter in many respects. The filtration method was easy, rapid and safe in its process and high in recovery and purity, while the differential centrifugation was laborious with low recovery and high contamination. We therefore determined the use of a polycarbonate membrane filter for the subsequent preparations of *Toxoplasma* antigen.

Survey of *Toxoplasma* antibody performed in this study showed 15.7 % of total prevalence rate in patients of our Hospital. Although it is difficult to make an exact comparison with previous reports on the survey in Japan (Kobayashi, 1977; Ise *et al.*, 1981) because of the difference in assay system and/or age distribution of sample population, the prevalence rate in ages of more than 20 years in our prefecture (calculated to be 18.5 %) was roughly higher than that in our neighbor, Osaka Prefectuer (9.3 %), and was lower than that in Tokyo (29.7 %). The prevalence among pregnant women in Tokyo (25.3 %, Kobayashi *et al.*, 1974; 21.3 %, Ise *et al.*, 1981) was also higher than that of corresponding population in our prefecture (7.1 % in female of 20-40 years). Because our previous comparison indicated that 34 positive sera were found by ELISA in 98 sera where the dye and latex agglutination tests resulted in 33 and 35 positive cases, respectively (Konishi and Takahashi, 1983), there seems to be a minor difference in prevalence according to the assay system.

Male population was infected at a higher rate than female population in all age groups but 10-19 and 60-69 years, and the difference of total prevalence was statistically significant ($p<0.001$). Similar results were obtained in other surveys performed in Hyogo Prefecture, which also indicated significantly higher prevalence in male (unpublished data). However, in contrast to sex and age difference in prevalence, mean antibody levels in positive groups were almost the same for male and female and for all age groups with one exception (10-19 years).

The frequency distribution did not show a typically bimodal pattern which was obtained in populations of healthy people (unpublished data). It is probably due to the high mean ELISA value of the negative group that increased with age. Further comparison with other surveys from various aspects will be necessary for more close analyses of *Tosoplasma* prevalence among inhabitants in Hyogo Prefecture.

Summary

The antibody to *Toxoplasma gondii* was surveyed among 1,331 patients of our Hospital in Hyogo Prefecture, Japan, as an approach to reveal the current situation of the prevalence. The assay system for antibody levels in sera was a magnetic processing enzyme-linked immunosorbent assay. In this study, tachyzoites used for antigen preparation were collected by the filtration method with a polycarbonate membrane, which was easy, rapid and safe in its process and high in recovery and purity. The prevalence rate was 15.7 % in a total population and it de-

pended on age of patients; 4.1-5.5 % in 1-29 years population and 13.3 % in 30-39 years, followed by a gradual increase with age and a mixiumum of 37.5 % in 80-93 years. In all age groups but 10-19 and 60-69 years, the male population was infected at a higher rate than the female population and the difference of total prevalence (20.3 % in male and 13.2 % in female) was statistically significant ($p<0.001$). However, in spite of the sex and age difference in prevalence rate, comparable mean antibody levels in positive groups were obtained for male and female and for all age groups with one exception (10-19 years). A typical bimodal pattern of frequency distribution of antibody levels was not observed, probably due to high mean antibody levels in negative groups of the population involved in this survey.

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兵庫県の病院患者を対象とした酵素抗体法による トキソプラズマ抗体保有調査

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兵庫県住民におけるトキソプラズマ（Tp）抗体保有状況を調査する一環として，神戸大学付属病院の患者1,331人を対象として酵素抗体法による血清中の抗体測定を行った．本研究で用いたTp抗原は，Nuclepore メンブランを用いた沪過法により得られた虫体から作成したもので，マウス細胞の混入は0.1％以下であった．抗体保有率は全体として15.7％であり，年齢別にみると1～29歳で4.1～5.5%，30歳代で13.3%，40歳代で20.1％と年齢に依存して上昇し，80～93歳では37.5%に達した．また男女別にみると，10歳代，60歳代を除く各年齢層で男の抗体保有率が高く，全体で男（20.3%）が女（13.2%）より有意に高かった（$p<0.001$）．一方，抗体陽性者の保有抗体量は10歳代を除くすべての年齢層で，また男女共ほぼ同値であった．抗体量の頻度分布は典型的な2峰性を呈さなかったが，おそらく陰性集団における高い抗体保有量に起因しているものと思われる．