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Enzyme-linked Immunosorbent Assay (ELISA) on Japanese Encephalitis Virus. I. Basic Conditions of the Assay on Human Immunoglobulin

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Abstract : Microplate ELISA was applied to estimate human immunoglobulin against Japanese encephalitis (JE) virus utilizing purified JE vaccine preparation as antigen in the test. Comparison of the optical density observed at a certain dilution of given test sera with those of serial dilutions of a standard positive serum gave the estimation on the ELISA end-point titer of the test sera obtained by their serial dilutions. Availability of the test system to large scale examination on the serum specimens was discussed.

INTRODUCTION

The principle of enzyme-linked immunosorbent assay (ELISA) as developed by Engvall and Perlman (1971) has been applied to detect the etiological agents or their antigens of various infectious diseases and also to assay antibody titers against these antigens (Voller *et al.*, 1976 ; Sever and Madden, 1977). Although ELISA has been reported as simpler, more rapid and sensitive than the conventional serological tests such as complement-fixation (CF) or hemagglutination-inhibition (HI) in many virus systems, there have been only a few reports on its application to arboviruses (Hofmann *et al.*, 1979 ; Frazer and Shope, 1979 ; Dittmar *et al.*, 1979) including JE virus (Miyata *et al.*, 1981). In this report, we examined several parameters of ELISA which will be necessary to quantitate human immunoglobulin against JE virus, and devised a simple method which could be used as a routine test.

MATERIALS AND METHODS

Antigen : Formalin-inactivated, purified JE vaccine concentrate (Takaku *et al.*, 1968) was kindly supplied by the Kanonji Institute, Research Foundation for Microbial

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Diseases of Osaka University. The original solution contained 2.8mg of protein per ml as determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Test sera : Four-hundred and fifty two human serum specimens were obtained from patients clinically diagnosed or suspected of JE during the epidemic season from 1965 until 1975. These sera have been preserved at −30°C in a refrigerator room of this Institute. Eight sera from JE patients in the year of 1978 were kindly supplied from Nagasaki Prefectural Institute of Public Health and Environmental Sciences. As negative control sera, specimens were collected from Africans living around Nairobi in Republic of Kenya, where JE is not endemic.

ELISA procedures : The procedures were slightly modified from those described by Voller *et al.* (1976). The plastic Immulon U-microplate (obtained from Sanko Pure Chemical Co. Tokyo) was coated with the antigen diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6, by distributing 100μl of the antigen solution in each well and incubating the plate in a refrigerator overnight. The wells on the plate were emptied and were washed with PBS-T (0.05% Tween 20 in phosphate-buffered saline, pH 7.2) 3 times for 3 minutes each. Test or standard sera diluted in PBS-T, were added to the plate using 100μl/well and the plate was incubated at 37°C for 1 hour. The plate was emptied and were washed as above. Peroxidase-conjugated anti-human immunoglobulin diluted in PBS-T was applied using 100μl/well and the plate was incubated at 37°C for 1 hour. The plate was emptied and washed as above. Substrate solution containing 0.5mg of *o*-phenylene diamine per ml and 0.02% of H_2O_2 in 0.05 M citrate-phosphate buffer, pH 5.0, was applied using 100μl/well and the plate was incubated at room temperature in the dark. The reaction was stopped by adding 75μl of 4N H_2SO_4 in each well and the optial density (OD) at 500 nm was recorded *in situ* by Corona double-beam spectrophotometer using 600 nm as a reference wavelength.

Reagents : Horse-raddish peroxidase-conjugated anti-human immunoglobulins (IgA +IgG+IgM, heavy and light chains) was the product of Cappel Laboratories, Pa. USA. *o*-Phenylenediamine dihydrochloride was obtained from Wako Pure Chemicals Co. Osaka.

RESULTS

Determination of antigen concentration : Microplate wells were coated with several dilutions of antigen to contain various protein concentrations. After overnight sensitization and washing, they were reacted with serial dilutions of a standard positive serum with HI titer of 1280 and of a standard negative serum. Peroxidase conjugate at 1:400 dilution was used in the test and the OD were recorded as shown in Fig. 1. High concentration of antigen with 100 or 200μg protein per ml gave high background OD with negative serum although they also gave high OD with positive serum, and low concentration of antigen with 5 to 10μg protein per ml gave low OD with positive serum. The

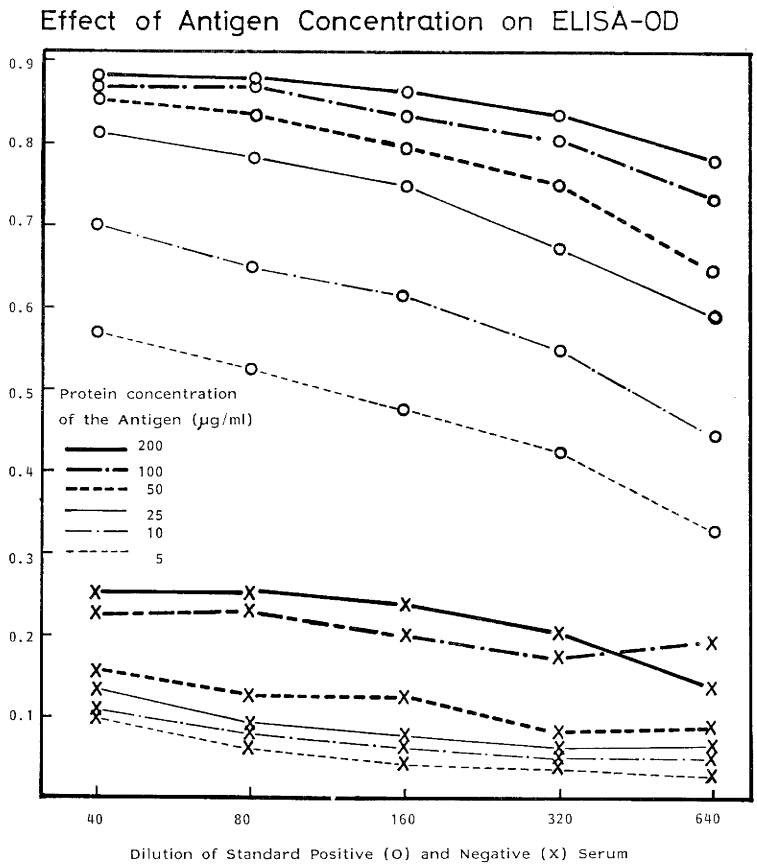

Fig. 1. Effect of antigen concentration on the ELISA-OD developed by serial dilutions of the standard positive and the negative serum (1). Microplate wells were coated with JE antigen serially diluted by 2-fold steps in order to contain various amounts of proteins as indicated in the figure. The plate was reacted with serial dilutions of the standard positive (open circle) and negative serum (x-mark), followed by the peroxidase-conjugate and the substrate reaction to develop the color reaction.

results of similar experiment using 3 dilutions of the antigen between 12.5 to 50μg protein per ml were shown in Fig. 2. Antigen concentration at 25 to 50μg protein per ml gave high signal to noise ratio and this condition was used in the following experiments.

Determination of conjugate concentration : Microplate was coated with antigen solution at 25μg protein per ml and was reacted with several dilutions of the standard positive and negative sera. The plate was then reacted with serial dilutions of the conjugate serum starting from 1:40 dilution and the OD readings were plotted in Fig. 3. Here again, higher concentration of the conjugate gave higher OD both by the positive and

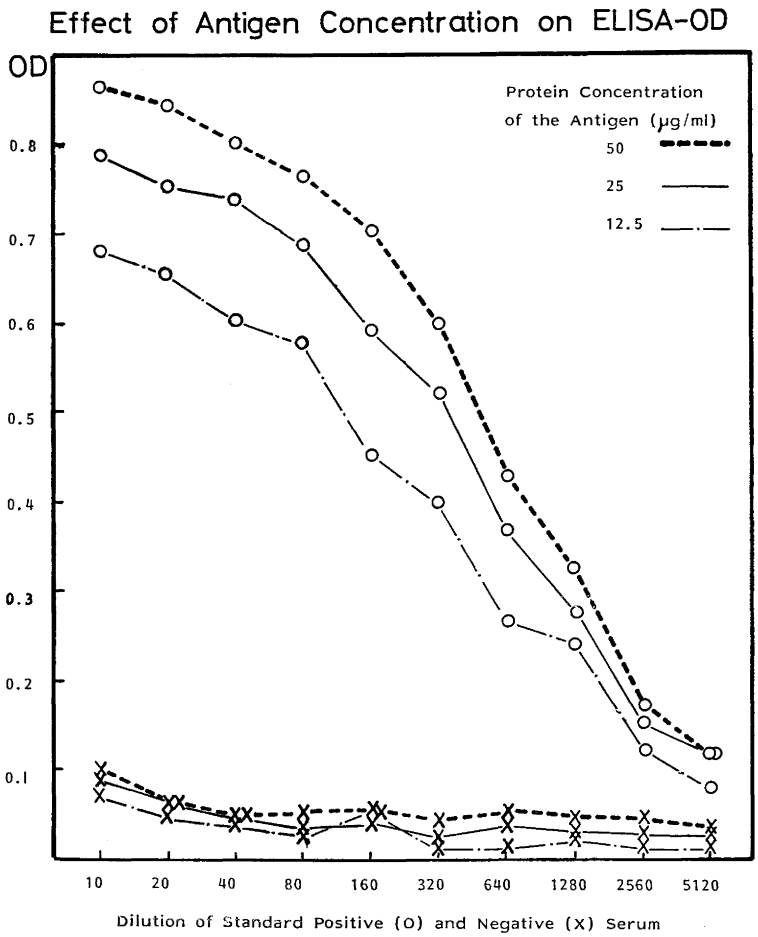

Fig. 2. Effect of antigen concentration on the ELISA-OD developed by serial dilutions of the standard positive and the negative serum (2). The methods are similar to those described in the text and in the legend to Fig. 1, using 3 different antigen concentrations and higher dilutions of the standard sera.

the negative sera, and the lower concentration gave lower OD. By conjugate dilutions between 1:320 to 1:640, OD at 1:100 dilution of the positive serum did not differ so much with that by 1:40 dilution of the conjugate with fairly low background value by the negative serum. Thus, the conjugate concentration at 1:400 dilution was used in the following experiments.

Determination of the peroxidase reaction time : Replicate wells on a microplate were coated with antigen at 25μg protein per ml and were reacted with the standard positive and the negative serum at 1:100 and 1:500 dilutions followed by the conjugate serum at 1:400 dilution. At various time intervals after the peroxidase reaction mixture

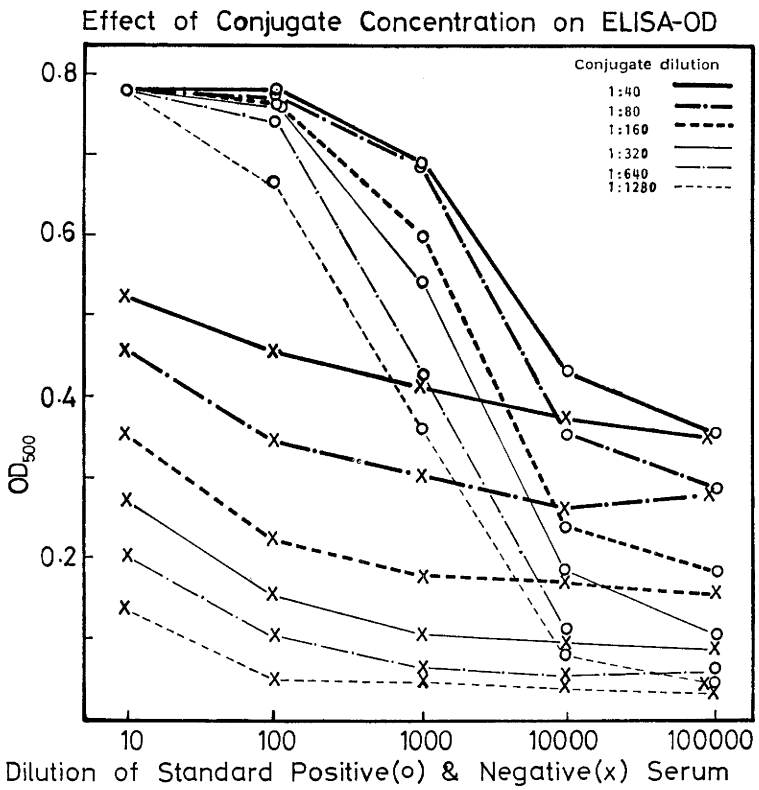

Fig. 3. Effect of conjugate concentration on the ELISA-OD developed by several concentrations of the standard positive and the negative serum. Plates were sensitized by the antigen (25 μg/ml) and were reacted with several dilutions of the standard positive (open circle) and the negative (x-mark) serum. Peroxidase-conjugate at various dilutions were used to develop the color reation.

was added, the reaction was stopped and the OD was recorded. Fig. 4A shows the difference between the ODs by the positive and the negative serum plotted against the peroxidase reaction time. The reaction appeared to proceed rapidly in the initial 5 minutes then gradually until 50 minutes. Fig. 4B shows the ratio of the OD by the positive and the negative serum plotted against the reaction time. The ratio, although high at the beginning, fell down to a certain level and stabilized after 60 minutes of incubation. Taking into consideration of the stability and reproducibility of the test, peroxidase-reaction time of 60 minutes at room temperature was chosen as the standard condition in the following experiments.

Examination of bovine serum albumin as a reagent to reduce "nonspecific" reactions : Several people employed bovine serum albumin (BSA) to reduce the nonspecific reactions caused by the binding of antisera or conjugate to the empty surface on the

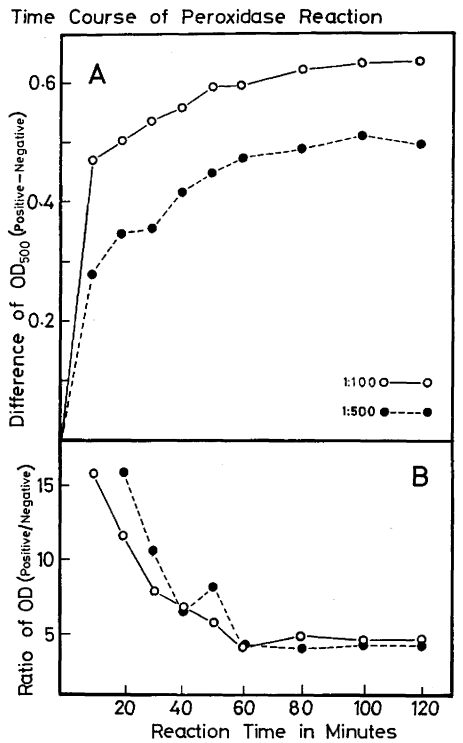

Fig. 4. Time course of the peroxidase reaction in the ELISA test. Methods are described in the text using 2 dilutions of the standard serum at 1: 100 (open circle and solid line) and 1:500 (closed circle and broken line), (A) Difference between the OD by the positive serum and that by the negative serum. (B) Ratio of these 2 OD values (positive/negative).

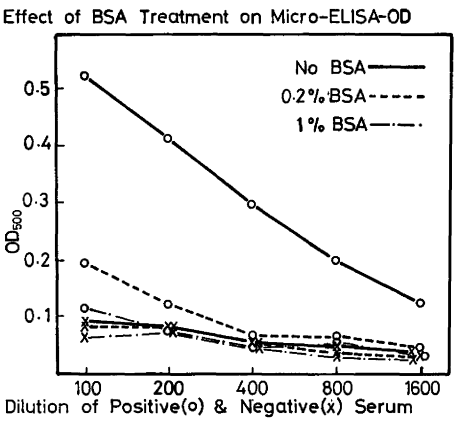

Fig. 5. Effect of bovine serum albumin (BSA) on the ELISA-OD. Microplate wells were sensitized with antigen (25 μg/ml) followed by treatment with BSA solution (1%, 0.2%, or 0%), and then reacted with serial dilutions of the positive or negative serum.

wells not covered by the antigen molecules. In our initial tests we introduced the BSA treatment, however, the color reaction was quite low and could not give reproducible results in the ELISA tests. Omission of the BSA treatment greatly improved the color reaction and one of the example is shown in Fig. 5. When the microplate wells were treated with BSA solution at various concentrations after the antigen coating, the color reaction decreased with higher concentration of BSA, and the reaction with negative serum was not appreciably reduced. Thus, BSA treatment was omitted from our system.

Estimation of the ELISA end-point titer from the OD obtained at a single dilution of the test sera: Microplates were coated with antigen solution of 25μg/ml of protein and then reacted with serial 2 fold dilutions of the standard positive and the negative sera, followed by the conjugate reaction at 1:400 dilution. The result of the OD readings were plotted in Fig. 6 in a thick-solid line and a thick-broken line, respectively. If the end-point of the ELISA reaction is taken as the reciprocal of the highest dilution of the test serum which shows more than twice the OD of the negative serum at the same dilution, this standard positive serum has the ELISA end-point titer of 10240. If we

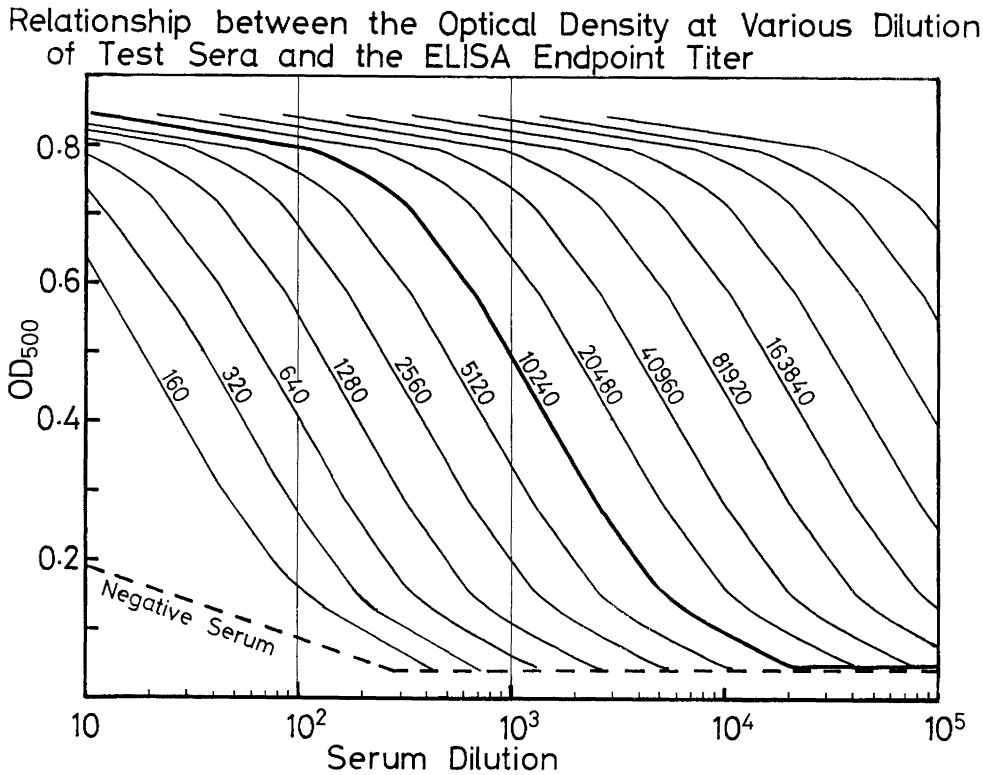

Fig. 6. Relationship between the OD at vaious dilutions of test sera and the ELISA end-point titer. Serial 2-fold dilutions of the standard positive serum (thick solid line) and the negative serum (broken line) were tested in the Micro ELISA to obtain their OD. ELISA end-point titer of 10240 was obtained for the standard positive serum. Dose-response ELISA-OD curves were depicted for several propored sera with various ELISA end-point titers, assuming that their curves follow similar pattern as that of the standard positive serum.

assume that the dose-response curve of the ELISA reaction with other sera follows the similar curve as the standard positive serum, they could be shown as the thin-solid lines parallel to the thick-solid line of the standard positive serum. Optical density of these lines at 1 :100 and 1 :1000 dilutions were read in the Fig. 6 and were replotted in Fig. 7 against the ELISA end-point titers of these supposed sera, giving the standard OD curve of the ELISA reaction. Practically this standard curve could be obtained by the color reaction of serial dilutions of the standard positive serum. Using this standard curve, one can theoretically estimate the ELISA end-point titer of a given serum by comparing its OD at 1 :100 or 1 :1000 dilution with the OD on the standard curve. Fox example, when the OD at 1 :100 dilution of a given serum is 0.6, its ELISA end-point titer is 1600, and when the OD at 1 :1000 dilution is 0.6, the ELISA end-point titer is 16000.

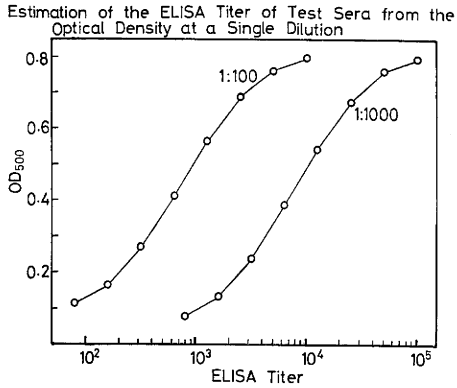

Fig. 7. Standard ELISA-curve for the estimation of the ELISA end-point titer of the test sera from the ELISA-OD at their single dilutions. OD values at 1:100 and 1:1000 dilutions of the proposed sera in Fig. 6 were replotted against their ELISA end-point titers.

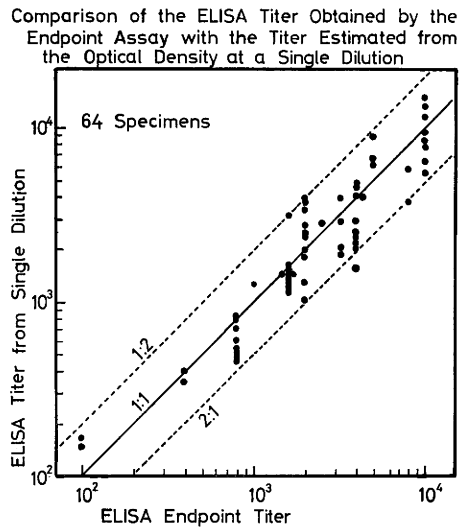

Fig. 8. Comparison of the ELISA end-point titer obtained by serial dilution of the test sera with the titer estimated from the ELISA-OD at a single dilution. Each dot represernt each of the 64 individual patient sera examined by the micro ELISA.

In the following studies, the standard curve was drawn every time on each plate using the serial dilution of the standard positive serum, so that the OD of given test sera could be compared with the standard curve on the same plate in order to eliminate any effects of the variables that may result in the difference in the color reaction. Photo 1 shows an example of such color reaction developed by a serial 2-fold dilution of the standard positive serum. Using 64 individual sera, their ELISA end-point titers were obtained (1) directly from the serial dilutions of the test sera and comparing their ELISA ODs with those of the serial dilution of the standard negative serum, taking the endpoint as the reciprocal of the highest dilution of the test sera that gives more than twice the OD of the standard negative serum at the same dilution, and (2) their ELISA endpoint titers were estimated by comparing the ELISA OD at 1:100 or 1:1000 dilution with the ODs of the serial dilution of the standard positive serum. The result plotted in Fig. 8 shows that these 2 values obtained by 2 different methods agreed quite well within the error of 2-fold except 4 specimens which showed slightly larger difference than 2 fold from each other.

Reproducibility of the test: Duplicate or triplicate specimens of 12 individual JE patients were examined by the micro-ELISA on 3 different days by the procedure described above in order to obtain their ELISA end-point titers from ELISA OD at a single dilution. The results shown in Fig. 9 indicate that the test has quite a good reproducibility and all the estimated titers fell within the range of 2-fold from their

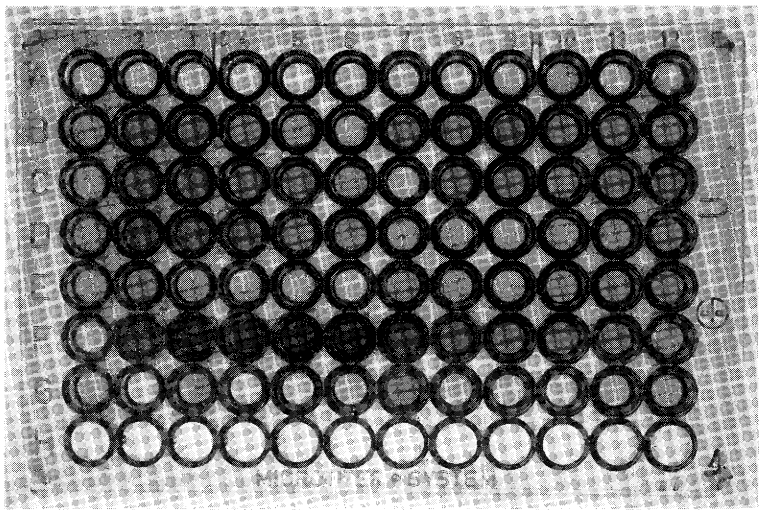

Photo 1. Color reaction of the Micro ELISA test. Lines E and F: serial dilutions of the standard negative and positive serum, respectively.

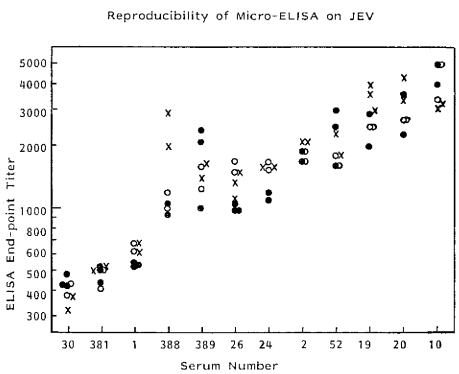

Fig. 9. Reproducibility of the ELISA test. Duplicate or triplicate specimens of 12 individual JE patient sera were tested on 3 different days (○, ●, ×) to obtain their ELISA end-point titers from the ELISA-OD at a single dilution.

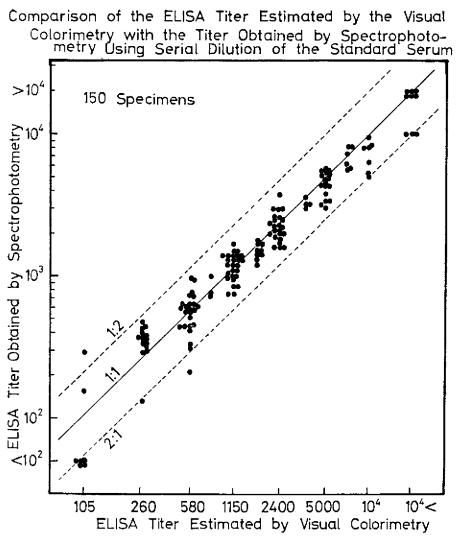

Fig. 10. Comparison of the ELISA titer estimated by visual colorimetry with the titer obtained by spectrophotometry using a single dilution of the test sera and serial dilutions of the standard serum. Each dot represent each of the 150 individual sera.

mean value for each serum.

Estimation of the ELISA end-point titer by visual colorimetry: The above mentioned tests were performed by reading in the spectrophotometer, however, comparison of the color reaction of the spcimens with the color reaction of the serial dilution of the standard positive serum could be performed visually. Hundred fifty individual serum specimens were tested by the ELISA to obtain their end-point titers from their single dilutions (1) by spectrophotometer, and (2) by visual colorimetry comparing the color reaction of the test specimen with the color reaction of the standard series of the positive serum. The results plotted in Fig. 10 indicate that these 2 values agreed very well for each serum specimen, so that the test could be performed in such an environment without sophisticated machine if standard positive serum of known end-point titer is available.

DISCUSSION

Japanese encephalitis is one of the serious viral diseases of great public health importance not only in Japan but also in other parts of Asia (Miles, 1960). In routine epidemiological or serological studies on JE, HI test has been widely used (Clarke and Casals, 1958; Oya and Okuno, 1967). Although ELISA has been reported as a simpler, more rapid and sensitive alternative to classical serological tests, most of the methods require serial dilution of the test sera with nagative and positive antigens in order to obtain their end-point titers. The method described in this report simplifies this problem giving the estimation on the ELISA end-point titers from a sigle or at most a few dilutions of the test sera using purified JE vaccine as antigen in the test. Thus, the test can be used more routinely with lower cost for many test materials. Also the result of visual colorimetry will give a chance to utilize the system in such laboratories or under the field conditions where spectrophotometer is not available.

Further studies will be necessary in order to apply the test to detest specific IgM or IgG antibodies and also to examine cross reactivity of the JE antigen with other flavivirus antibodies.

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日本脳炎ウイルスに対する免疫酵素測定法 (ELISA). Ⅰ. ヒト免疫グロブリン測定の基礎的条件.

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日本脳炎 (JE) 精製ワクチンを抗原として JE に対するヒト免疫グロブリンをマイクロプレート ELISA により測定した. 被検血清のある一定稀釈に対する吸光度を, 標準陽性血清の階段稀釈系列に対応する吸光度と比較する事によって, 被検血清の階段稀釈系列から求められる ELISA 終末力価を推定することが出来た. この方法によれば多数の検体を容易に検査することが出来る.

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