Enzyme-linked Immunosorbent Assay (ELISA) on Japanese Encephalitis Virus. III. Assay on Antibody Titers in Swine Sera

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Abstract: Antibody titers against Japanese encephalitis (JE) virus were measured by indirect enzyme-linked immunosorbent assay (ELISA) using 330 sera from slaughtered swines in Nagasaki, 1981. Detection of anti-JE immunoglobulin (Ig) antibodies by the ELISA almost paralleled to that by the hemagglutination-inhibition (HI) and the titer by the ELISA was approximately 10 times higher than that by the HI. Presence of IgM class of anti-JE antibodies was detected in 97 specimens by the ELISA in contrast to 33 by the HI test using 2-mercaptoethanol (2ME) treatment. Sixty five of the 97 specimens were shown to possess IgM antibodies only by the ELISA, while there was a single specimen with demonstrable 2ME-sensitive HI antibodies and negative IgM-ELISA. Most of the specimens with positive IgM-ELISA and negative 2ME-sensitive HI antibodies were those with high-titered Ig antibodies found late in the epidemic season.

INTRODUCTION

Japanese encephalitis (JE) virus circulates in nature by biological transmission cycle between *Culex tritaeniorhynchus* and swines during epidemic seasons in Japan (Mitamura *et al.*, 1938; Hammon *et al.*, 1949; Buescher *et al.*, 1959; Scherer *et al.*, 1959; Matsuyama *et al.*, 1960; Konno *et al.*, 1966; Fukumi *et al.*, 1971). Antibody surveillance among swine population is routinely used as an indicator to know the spread of the virus along with virus isolation from vector mosquitoes (Oya and Okuno, 1972; Oya, 1978). Usually the surveillance has been performed by the hemagglutination-inhibition (HI) test combined with detection of 2-mercaptoethanol (2ME)-sensitive antibodies. Since enzyme-linked immunosorbent assay (ELISA) was introduced as a new serological techniques with several advantages (Engvall and Perlman, 1971), it has been applied to various systems of virus infections (Voller *et al.*, 1976; Sever and Madden, 1977). We have been trying to adapt the method to detect anti-JE antibodies in human

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sera (Igarashi *et al.*, 1981a; Bundo *et al.*, 1981). In this report, we describe application of the ELISA to measure anti-JE antibodies in swine sera collected from a slaughter house in Nagasaki Prefecture in the year of 1981.

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 Diseases of Osaka University. The stock solution contained 2.8 mg of protein per ml as determined by the method of Lowry *et al.* (1951) using bovine serum albumin fraction V (Armour, Ill. USA) as a standard. The solution was diluted in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) to protein concentration of 25 μg/ml for the assay of immunoglobulins (Ig) and to 100 μg/ml for IgM assay, respectively.

Test sera: Three hundred and thirty swine sera in Nagasaki were kindly supplied by Dr. R. Matsuo, Nagasaki Prefectual Institute for Public Health and Environmental Sciences, together with the data on their HI tests. The sera were collected from July 2 until September 17 almost at weekly intervals at a slaughter house in Isahaya City (32°50′N, 130°03′E) as described (Igarashi *et al.*, 1981b). Forty two swine sera collected in Hokkaido, JE-nonendemic northern island, were kindly supplied by Prof. N. Hashimoto, Hokkaido University, School of Veterinary Medicine.

Sucrose gradient sedimentation: Separation of immunoglobulins of IgM and IgG classes was performed as described by Caul *et al.* (1974) or Vesikari and Vaheri (1968). One-half ml of serum specimen diluted 1 : 5 in PBS (phosphate-buffered saline by Dulbecco and Vogt, 1954) was layered on top of a 4.5 ml sucrose gradient (10−40 % in PBS) and was centrifuged at 35,000 rpm for 16 hours at 4°C. Twelve fractions were collected from the bottom of each tube after the run. One-tenth ml of each fraction was mixed with 0.3 ml of 0.13 M 2ME (final concentration of 2ME was 0.1 M), and was incubated at 37°C for one hour. Control specimen from each fraction was mixed with PBS and was run in parallel. The treated specimens were then extracted with cold acetone, restored by borate-buffered saline before being tested by the HI as described by Clarke and Casals (1958) using microtiter system. Assay on Ig- and IgM-ELISA was performed as follows.

ELISA procedures: Indirect method using microplate (Voller *et al.*, 1976) was followed with slight modifications (Igarashi *et al.*, 1981a; Bundo *et al.*, 1981). The method is referred as ordinary 2-steps method, in contrast to "3-steps method" which was introduced to measure IgM antibodies in sera of any species for which IgM-species enzyme conjugates were not commercially available. For the "3-steps method", a U-shaped plastic microplate (Immulon, Greiner Labortechnik, West Germany) was coated with diluted antigen, washed with PBS-T (PBS containing 0.05 % of Tween 20 and

0.01 % NaN_3), and then reacted with test sera diluted in PBS-T, similarly to ordinary 2-steps method. The plate was washed with PBS-T and then reacted with rabbit IgG with appropriate class and species-specificity against antibodies to be assayed. The plate was incubated at 37°C for one hour, washed with PBS-T, and then reacted with peroxidase-conjugated anti-rabbit IgG goat IgG diluted 1 : 1000, followed by washing and peroxidase reaction as in the case of ordinary 2-steps method. Volumes of the reactants were 100 μl/well except that the stop solution of 75 μl of 4 N H_2SO_4.

Reagents: Peroxidase-labelled anti-swine IgG rabbit IgG (heavy and light chain specific) and peroxidase-labelled anti-rabbit IgG goat IgG were obtained from Cappel Laboratories, Pa. USA. Anti-swine IgG (whole molecule) and anti-swine IgM (μ-chain specific) were the products by Miles Laboratories, Ind. USA. *O*-Phenylenediamine dihydrochloride was obtained from Wako Pure Chemicals Co. Osaka.

Statistical methods: Methods described in the textbook by Snedeco (1952) were followed.

RESULTS

Comparison of "3-steps method" with ordinary 2-steps method of indirect ELISA: Sixty swine sera with positive HI titers were tested by the ELISA using "3-steps method" and ordinary 2-steps method as described in the Materials and Methods. In the "3-steps" method, antigen-coated plate was reacted with test sera diluted 1 : 100 or 1 : 1000 followed by the reaction with anti-swine IgG rabbit IgG diluted 1 : 1000, and then peroxidaseconjugated anti-rabbit IgG goat IgG diluted 1 : 1000. While, in ordinary 2-steps method, reaction with test sera was followed by the reaction with peroxidase-labelled anti-swine IgG rabbit IgG diluted 1 : 1000. After the peroxidase reaction and additionof H_2SO_4, color density was measured by a microplate spectrometer (Corona 2-wavelength), and the titers of test sera were estimated from their ELISA-OD with those developed by serial dilutions of a standard positive serum as in the case of human sera (Igarashi *et al.*, 1981a; Bundo *et al.*, 1981). Fig. 1 compares titers obtained by these 2 methods for each of 60 individual swine sera. High correlation was observed between the logarithm of the titers with correlation coefficient of 0.96 ($p<0.001$) and equation of linear regression of $Y=0.77X+0.66$. In the following results, values obtained by the "3-steps method" were shown.

Sucrose gradient sedimentation of swine sera: Several swine sera with relatively high HI and demonstrable 2ME-sensitive antibodies were fractionated by sucrose gradient sedimentation in order to separate IgG and IgM class of antibodies as described in the Materials and Methods. Each fraction was assayed for its HI with and without 2ME treatment. Also, ELISA titers in each fraction was measured by the "3-steps" method using anti-swine IgM rabbit IgG (μ-chain specific) or anti-swine IgG rabbit IgG (whole molecule), both at 1 : 1000 dilution. As shown in Fig. 2, 2-peaks of anti-JE antibodies

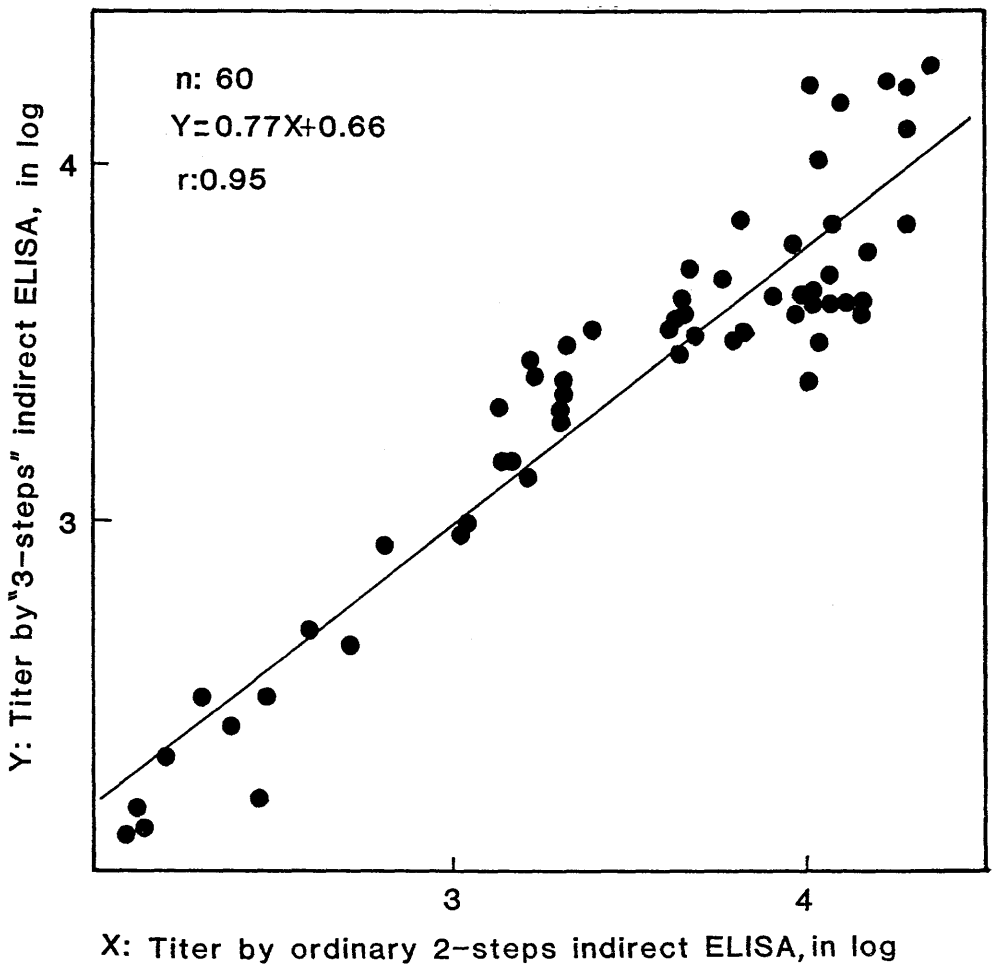

Fig. 1. Relationship between the ELISA titer assayed by "3-steps method" and that by ordinary 2-steps indirect method. Each of the 60 swine sera with positive HI tiers was assayed for its Ig-ELISA titer by the "3-steps method" and by ordinary 2-steps indirect method as described in the Materials and Methods and in the text.

were observed both by the HI and ELISA using anti-swine IgG (Panel A and B). The faster sedimenting peak in fractions 3 and 4 corresponded with fractions containing human IgM, and the slower sedimenting peak in fractions 7 and 8 with those containing human IgG, which were run in a parallel gradient, respectively. Since, the anti-swine IgG rabbit IgG was raised against whole molecule of swine IgG, it reacted with both IgG and IgM class of antibodies through their light chains. Therefore, the ELISA titer assayed by the 3-steps method using this anti-swine IgG was henceforth called Ig-ELISA. However, anti-JE antibody titers in fractions 3 and 4 decreased after 2ME-

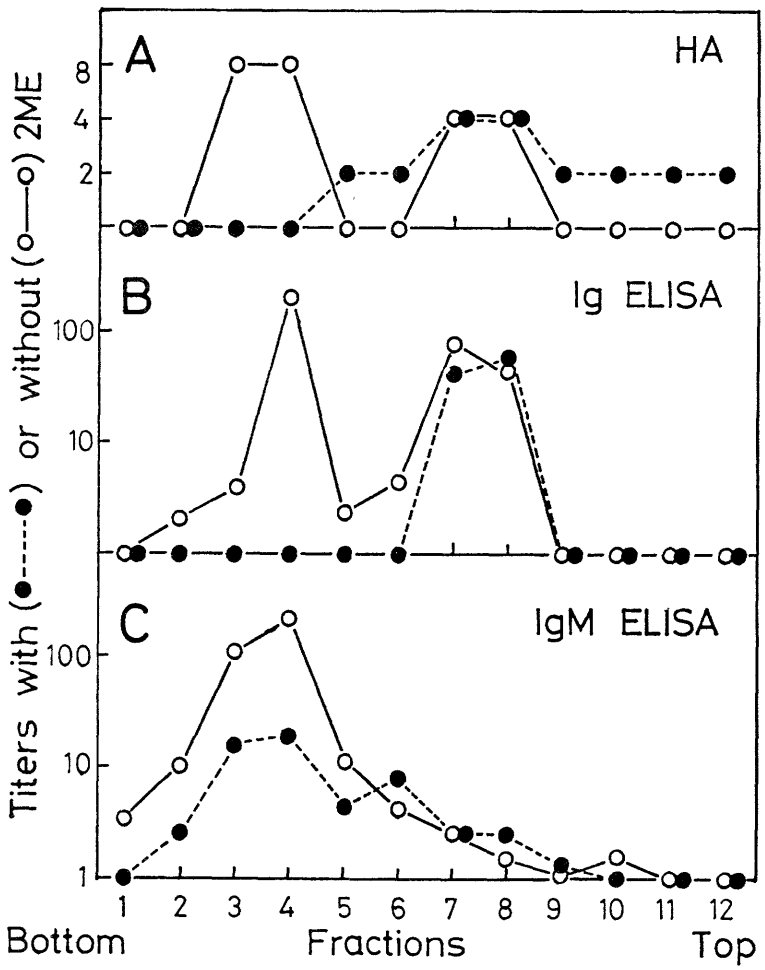

Fig. 2. Fractionation of swine serum by sucrose gradient velocity sedimentation. Swine serum with HI titer of 320 was diluted 1:5 in PBS and was fractionated as described in the Materials and Methods. HI titer of the serum after 2ME-treatment was less than 10.

treatment. Results with anti-swine IgM (μ-chain specific) rabbit IgG is shown in Panel C. The ELISA titer was observed only in and around fractions 3 and 4 and decreased after 2ME-treatment. The results support our assay system of "3-steps method" showing that by using μ-chain specific anti-swine IgM rabbit IgG, it can measure antibody titers of IgM class in swine sera, even when such class-specific enzyme conjugate is not available.

Comparison of Ig-ELISA titer with HI titer: Three hundred and thirty Nagasaki swine sera were tested for their HI and Ig-ELISA titers and the results with 126 specimens with positive HI titers ($\geqq$10) were plotted in Fig. 3. Correlation was observed

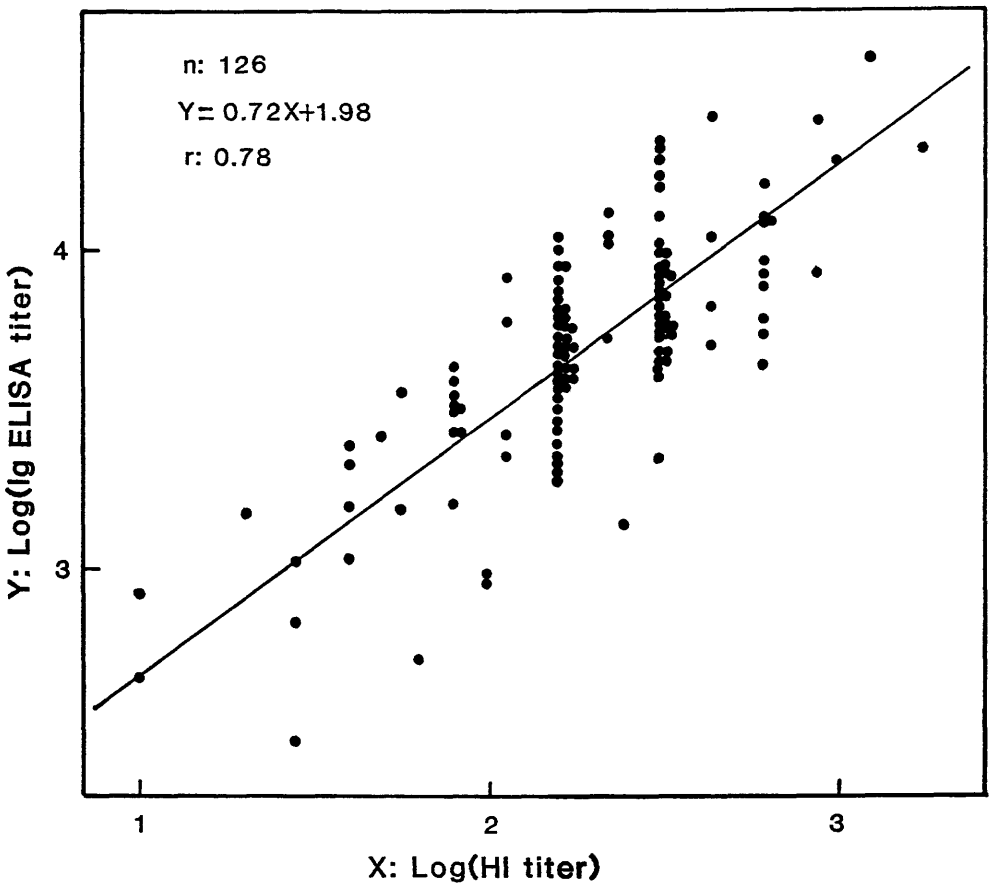

Fig. 3. Relationship between the Ig-ELISA and HI titers of HI-positive Nagasaki swine sera. Each of the 126 swine sera was assayed for its Ig-ELISA and the titer was compared with the HI.

between the logarithm of the HI (X) and that of Ig-ELISA (Y) titers with correlation coefficient of 0.78 ($P<0.001$) and equation of linear regression of $Y=0.72X+1.98$, showing that the Ig-ELISA was almost 10 times higher than the HI. For the remaining 204 specimens with negative HI (<10), geometrical mean titer (GMT) of Ig-ELISA was calculated to be 130 with range of distribution under 100 up to 492. When these 204 specimens were included in the calculation, the correlation coefficient between X and Y becomes 0.96 with equation of linear regression of $Y=0.93X+1.48$. The results indicate high degree of correlation bween the titers obtained by the Ig-ELISA and by the HI.

Comparison of IgM-ELISA with 2ME-sensitive HI antibody titers: For those 126 specimens with positive HI titers, IgM ELISA was performed by the "3-steps method" using anti-swine IgM (μ-chain specific) rabbit IgG, and the logarithm of the IgM-

ELISA titers (Y) was compared with the logarithm (X) of the ratio of the HI titers before and after 2ME-treatment (Fig. 4). Correlation between these 2 variables were relatively low with correlation coefficient of 0.45 ($P<0.001$) with equation of linear regression of $Y=0.51X+2.50$. The low correlation is probably due to the fact that X represents the ratio, rather than the absolute amount, of 2ME-sensitive HI in total HI antibodies. There were 33 specimens with the HI ratio (before and after the 2ME-treatment) over 8, and these specimens were considered to possess IgM antibodies according to the criteria by the National Institute of Health of Japan (Oya, 1978). All, but one, of the 33 specimens possessed IgM-ELISA titer over 300. While, there were 93 specimens with HI ratio under 8, and 65 out of the 93 showed IgM-ELISA titer

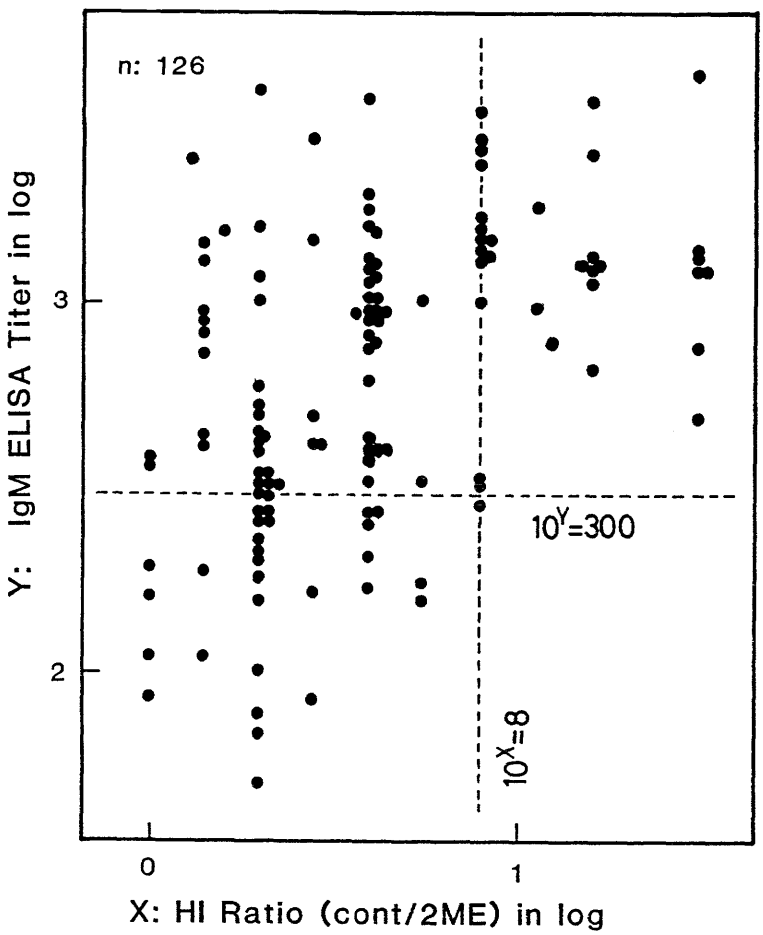

Fig. 4. Comparison of the IgM-ELISA titer with the ratio of HI titers before and after 2 ME-treatment of swine sera. Each of the 126 swine sera shown in Fig. 3 was assayedfor its IgM-ELISA and compared with its ratio of the HI titers before and after 2 ME-treatment.

over 300. When 204 specimens with negative HI (<10) titer were assayed for their IgM-ELISA, their GMT was calculated as 66 with range of distribution under 100 up to 261, and none of them showed IgM-ELISA exceeding 300.

Frequency distribution of ELISA titers: Results with Ig- and IgM-ELISA for these Nagasaki swine sera as well as for 42 Hokkaido specimens were shown in Fig. 5. All the specimens from Hokkaido (bottom panel) possessed Ig-ELISA titer under 400 and IgM-ELISA titer under 300. Also, all but one HI-negative 204 Nagasaki sera possessed Ig-ELISA under 400. This single specimen was taken on August 4 with Ig-ELISA titer of 492 and IgM-ELISA titer of 261, presumably showing early stage of infection. All the 204 HI-negative Nagasaki sera possessed IgM-ELISA titer under 300. There were 126 Nagasaki sera with positive (≧10) HI titers, and all but two of these specimens possessed Ig-ELISA titer over 400. One of the 2 specimens was taken on August 11 with HI titer of 28 and Ig-ELISA titer of 321, presumably showing early stage of JE infection. On the other hand, another specimen taken on August 11 possessed HI titer of 10 and Ig-ELISA less than 100 and this specimen could probably be considered as negative with some nonspecific HI activities. As described in the above section, there were 97 specimens with IgM-ELISA over 300, and 32 of them were shown to possess 2ME-sensitive HI antibodies. However, remaining 65 specimens were demonstrated to possess IgM antibodies only by the IgM-ELISA. In contrast, there was a single specimen, which showed positive 2ME-sensitive antibodies and relatively low IgM-ELISA titer of 282. As shown in Fig. 5, there were 8 specimens with Ig-ELISA over 12800, and none of them were shown to possess 2ME-sensitive HI antibodies. However, 7 of them showed IgM-ELISA over 300. There were 29 specimens with Ig-ELISA titer between 6400 and 12800, and 2 of them were shown to possess 2ME-sensitive HI antibodies, in contrast to 26 specimens with IgM-ELISA titer over 300. The results indicate higher efficiency to detect IgM class antibodies by the ELISA compared with conventional 2ME-treatment and the HI test. From these results we would like to propose a tentative criteria of detecting anti-JE antibodies in swine sera: positive Ig-ELISA titer over 400, and positive IgM-ELISA titer over 300.

Changes in the anti-JE antibodies in Nagasaki swine sera in the year of 1981: Because 330 Nagasaki swine sera were collected almost at weekly intervals taking 30 specimens at each collection, frequency distribution of the ELISA titers for each sampling date was shown in Fig. 6. In Fig. 7, changes in the antibody positive rate by the above criteria for ELISA and also for the positive HI titer over 10 is shown along with the data of virus isolation which were reported previously (Igarashi *et al.*, 1981b). Fig. 8 shows changes in the GMT of ELISA and HI, as well as the HI ratio (before and after 2ME-treatment). As reported previously, the first virus isolation in Nagasaki, 1981, was from *Culex tritaeniorhynchus* collected on July 27 (Igarashi *et al.*, 1981b). On the same day, one out of the 30 swine sera showed Ig-ELISA titer of 848, with HI

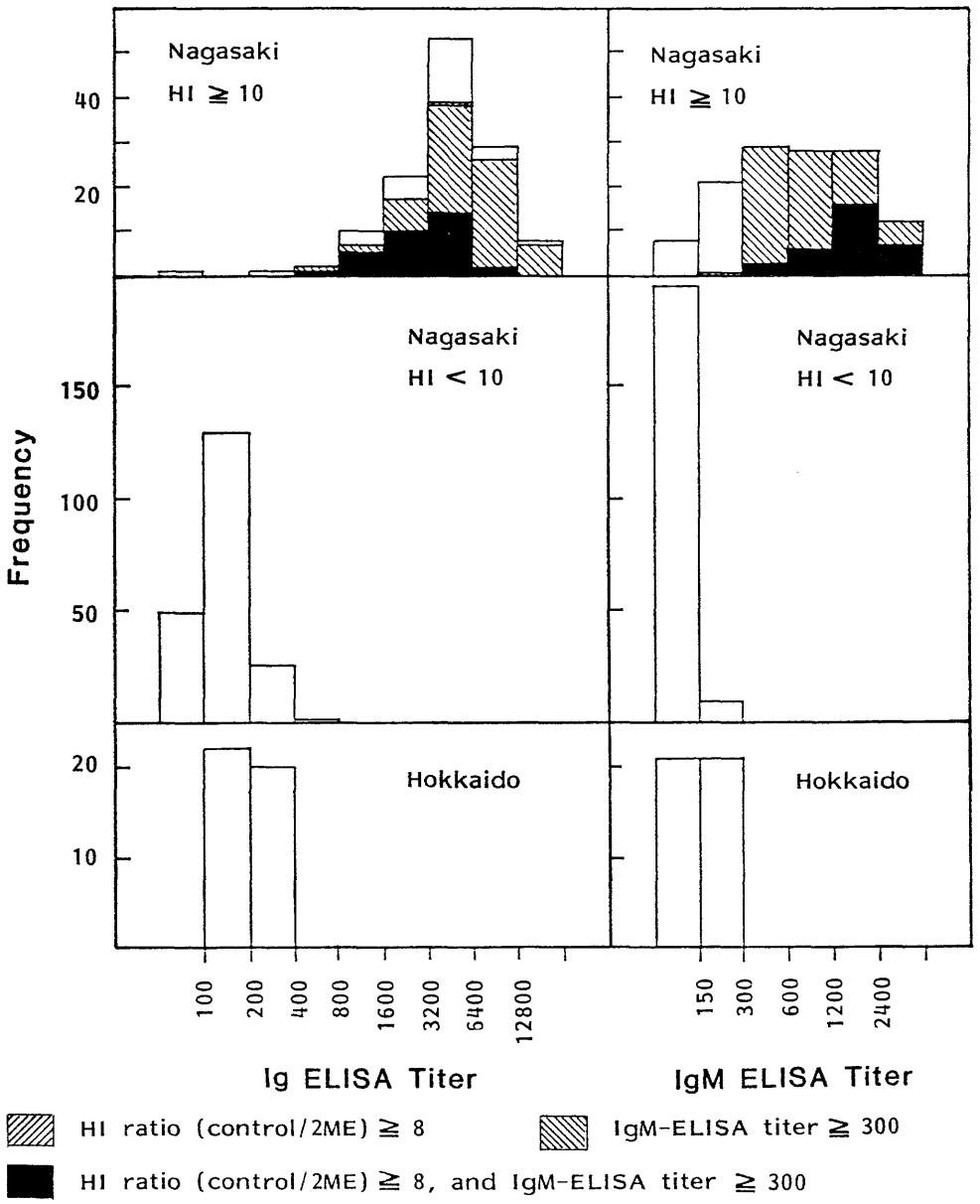

Fig. 5. Frequency distribution of ELISA titers of swine sera from Nagasaki and Hokkaido in the year of 1981. Swine sera from Nagasaki Prefecture with positive HI (upper panel), and negative HI (middle panel), and specimens from Hokkaido (bottom panel) were measured for their Ig-and IgM-ELISA titers and frequency distribution is shown.

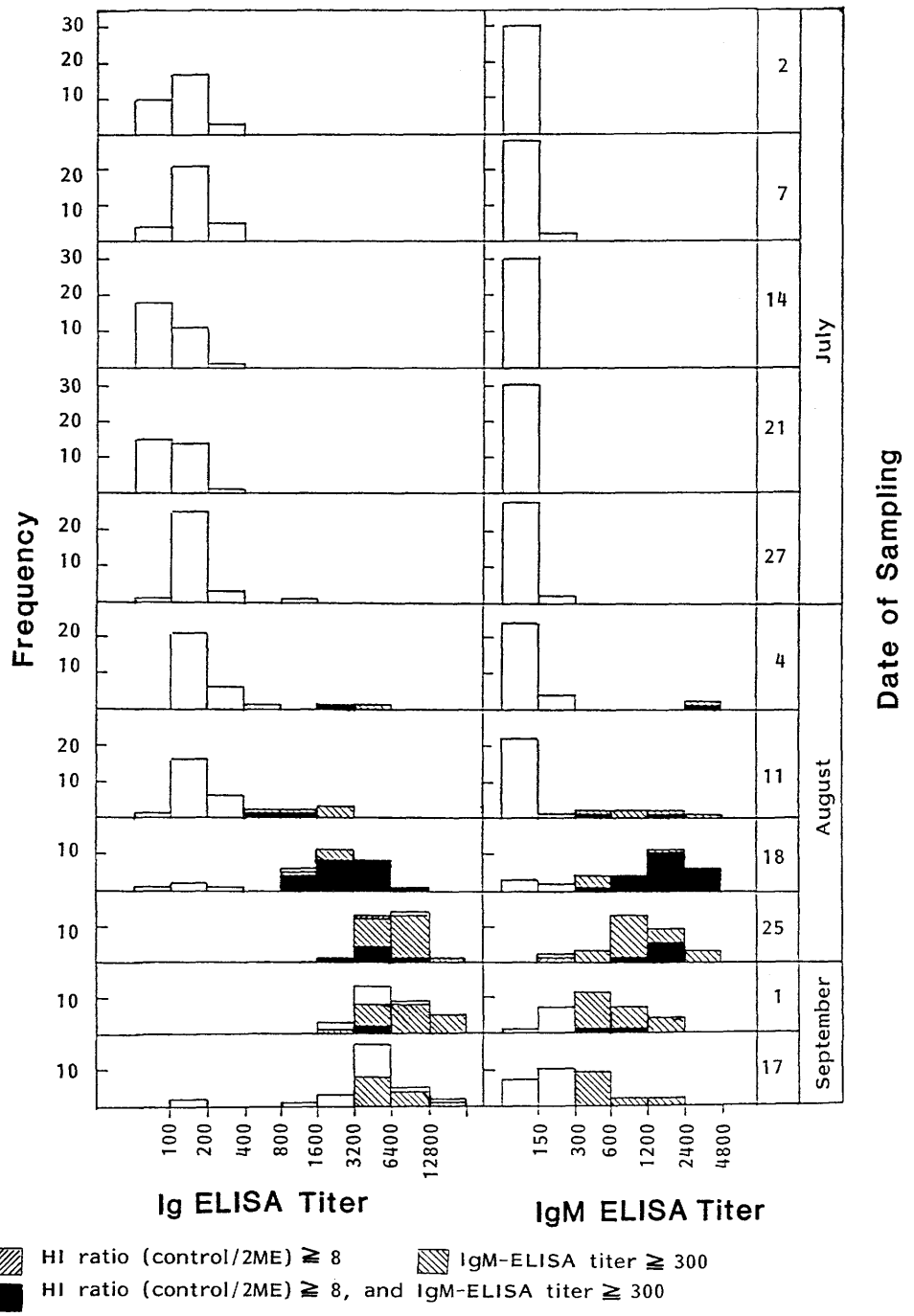

Fig. 6. Changes in the frequency distribution of ELISA of titers in sera from slaughtered swines in Nagasaki Prefecture, 1981. Each of the 330 swine sera obtained from a slaughter house in Isahaya City was assayed for its Ig-and IgM-ELISA titers and frequency distribution of the titers was shown according to the date of sampling.

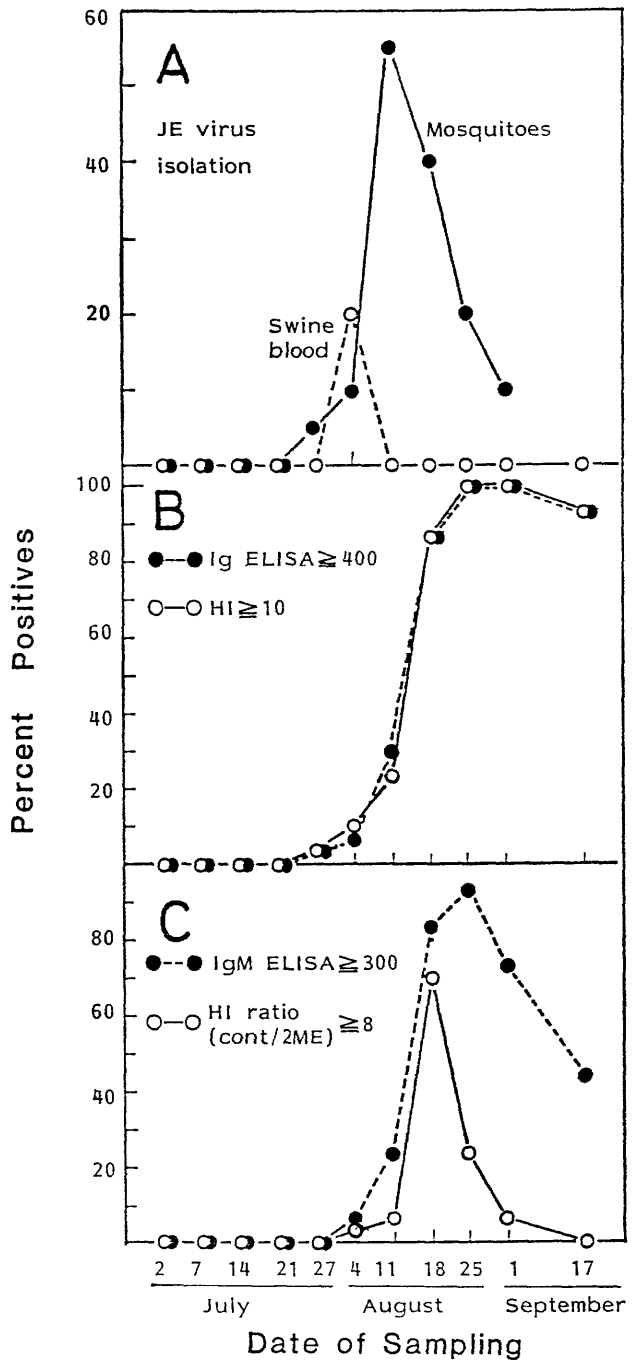

Fig. 7. Isolation of JE virus and changes in the antibody positive rates in sera from slaughtered swines in Nagasaki Prefecture, 1981. The data of JE virus isolation (Igarashi *et al.*, 1981b) was reproduced in panel A. Antibody positive rates as measured by the Ig-ELISA (over 400) and by the HI (over 10) is shown in panel B. panel C shows antibody positive rate of IgM-ELISA (over 300) and that by the HI test combined with 2 ME-treatment (HI ratio over 8).

titer of 10, however, its IgM-ELISA titer was less than 300. On August 4, JE virus was isolated from 6 out of 30 swine blood specimens (Igarashi *et al.*, 1981b) and 3 of the remaining 24 sera showed Ig-ELISA over 400, and 2 of them possessed IgM-ELISA titer over 300. However, only one of the 2 specimens was demonstrated to possess 2ME-sensitive HI antibodies. On August 11, infection rate of *C. tritaeniorhynchus* became highest, and 2 weeks later, antibody positive rate in swine sera became 100 % both by the Ig-ELISA and HI. On the other hand, positive rate of 2ME-sensitive HI

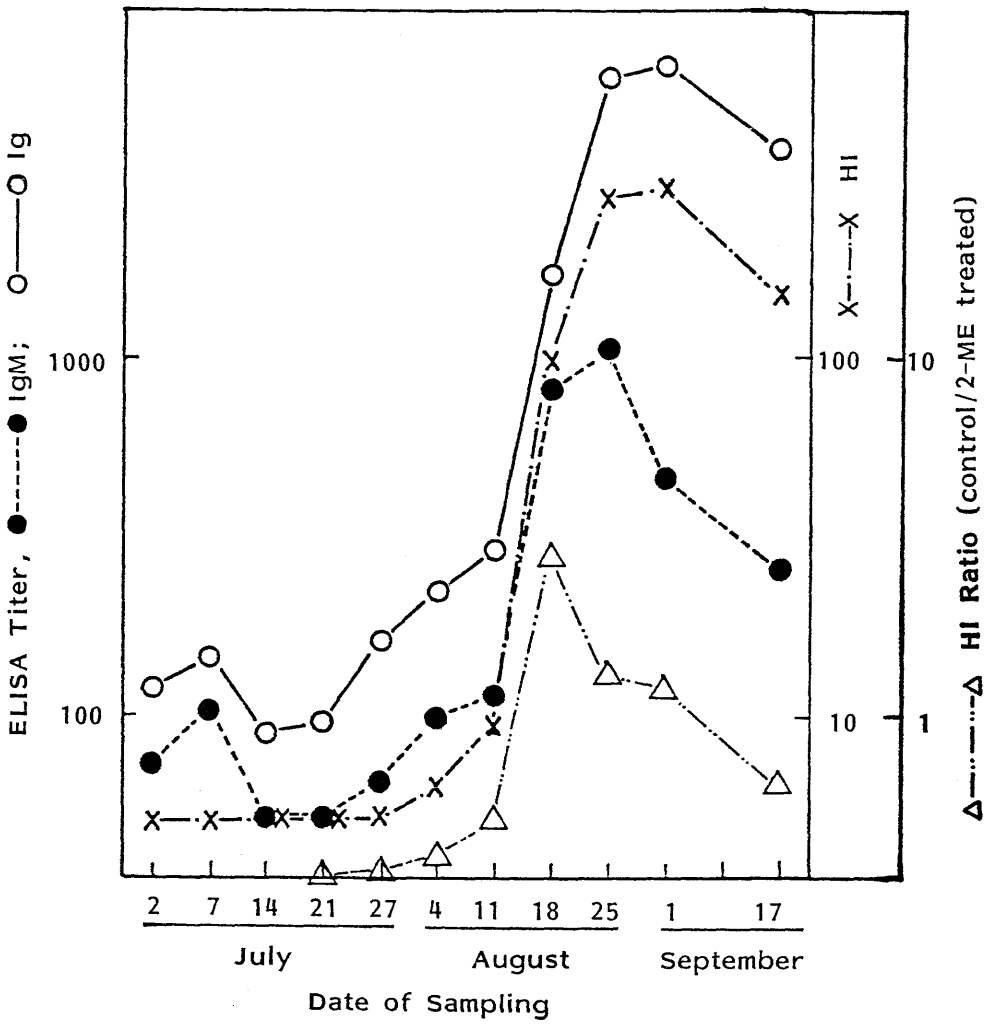

Fig. 8. Changes in the geometrical mean titer (GMT) of anti-JE antibodies in sera from slaughtered swines in Nagasaki, 1981. GMTs were calculated for Ig-ELISA, IgM-ELISA, and HI titers, as well as for the ratio of HI titers before and after 2 ME-treatment, and were shown according to the date of sampling of slaughtered swine sera.

antibodies became its peak (70 %) on August 18, followed by rapid decrease in the next week. However, positive rate of IgM-ELISA continued to increase from August 18 (83.3 %) to August 23 (93.3 %) followed by gradual decrease. The results again indicate that 2ME-treatment with the HI test is rather inefficient to detect IgM class on antibodies in those sera with relatively high IgG antibody titers, although it could detect IgM antibody at early stage of infection in the epidemic. As shown in Fig. 8, GMTs of Ig-ELISA and HI titers reached their plateau on August 25, with their maximum on September 1, in contrast to that of IgM-ELISA which reached plateau and peak almost one week earlier. The GMT of HI ratio (before and after 2ME-treatment) reached its peak on August 18 when highest numbers of the specimens showed positive 2ME-sensitive HI antibodies.

DISCUSSION

According to our tentative criteria of detecting ELISA antibodies (positive limit of Ig-ELISA of 400, and that of IgM-ELISA of 300), there were high degree of correlations between antibody-positive rate by the Ig-ELISA and by the HI. On the other hand, IgM-ELISA could detect almost twice as many specimens with probable IgM antibodies compared with the HI test and 2ME-treatment. Although the latter classical method could effectively detect IgM class of antibodies in the early stage of epidemic season, or possibly in the early stage of virus infection in individual swines, easiness and rapidity of the IgM-ELISA will be great advantages over the classical method of 2ME-treatment followed by acetone extraction and HI test especially for testing large numbers of specimens. Application of ELISA to JE and some other flavivirus antibodies in bovine sera has been reported by Miyata *et al.* (1981). Konishi and Yamaoka (1982), Konishi *et al.* (1982), and Takashima *et al.* (1982) also reported ELISA systems to measure anti-JE antibodies in swine sera. Although their method are somewhat different from ours, characteristics of our system are to estimate endpoint titer of test sera from their ELISA ODs at a single dilution by comparing with ODs by serial dilutions of a standard positive serum, resulting in reproducible titers. We are now introducing a computer system for this estimation with great saving of time consumed in previous graphical method. Similar assay was also used to measure anti-Getah virus antibodies. These results will be reported in separate papers. Attempts will be made to assay anti-dengue ELISA titers, trying to detect type-specific IgM 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）. Ⅲ. ブタ血清中の抗体価の測定.
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1681年の長崎県下のと場ブタ血清330検体を用い，日本脳炎（JE）ウイルスに対する抗体価を，免疫酵素抗体法（ELSA）の間接法により測定した．ELISA による抗 JE 免疫グロブリン抗体価の検出と血球凝集抑制法（HI）によるものは，ほとんど一致した．

抗 JE 抗体の IgM クラスは，2メルカプトエタノール（2ME）処理による HI法で，33検体が検出されたのに比べて，ELISA では97検体が検出された．97検体中，65検体は ELISA でのみ IgM抗体が認められたが，一方，2ME 感受性 HI 抗体が証明され，IgM-ELISA抗体が陰性のものが1検体あった．IgM 抗体陽性で2-ME 感受性 HI 抗体が陰性の検体の大部分は，流行の末期の高い抗体価の認められたものであった．

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