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# Antibodies against Japanese Encephalitis Virus in Bovine Sera in Nagasaki, 1981.

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**Abstract**: Antibodies against Japanese encephalitis (JE) virus in sera from bovines placed at 5 locations in Nagasaki Prefecture were measured by the hemagglutination-inhibition (HI), neutralization (N) and enzyme-linked immunosorbent assay (ELISA) during summer seasons in 1981. In every location, both the antibody positive rates and the geometrical mean titer (GMT) of antibodies were found to increase during the epidemic season, indicating the wide-spread infection of bovines by JE virus. Detection of JE virus infection was highest by the N, followed by the ELISA, then by the HI. Comparison of the antibody levels and antibody positive rates among bovines with those in swine indicated that bovines are less sensitive to JE virus compared with swine.

*Key Words*: ELISA, Japanese encephalitis virus, antibody, bovine

## INTRODUCTION

It is well known that swine play an important role in the circulation of JE virus as major amplifier vertebrate hosts (Scherer et al., 1959; Konno et al., 1966; Fukumi et al., 1971). On the other hand, bovines are considered generally less sensitive to JE virus, although they effectively attract vector mosquitoes, *Culex tritaeniorhynchus* (Otsuka et al., 1969; Matsuo et al., 1968).

As reported previously, some of the authors of this paper reported the isolation of JE virus from vector mosquitoes and slaughtered swine sera (Igarashi et al., 1981) and antibody prevalence among swine sera by the ELISA (Bundo et al., 1982) during epidemic seasons in Nagasaki, 1981. In relation to those data, we examined antibody prevalence against JE virus in sera from bovines placed at 5 locations in Nagasaki Prefecture in the same epidemic season, and also the applicability of the ELISA to seroepidemiological survey on bovines.

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## MATERIALS AND METHODS

*Test sera*: Three hundred and nineteen serum specimens were taken successively from approximately 20 bovines at each of the 5 locations (Iki, Gotoh, Saseho, Azuma, and Ohseto) as shown in Fig. 1. Sampling was performed almost once a month starting from May in Saseho, and from June in other places, ending in August in Iki, Azuma and Ohseto, and in September in Gotoh and Saseho. Sampling from Shimabara was performed in September and the data were used instead of the specimens from Azuma (Table 1). Sera were kept frozen at −20°C until use.

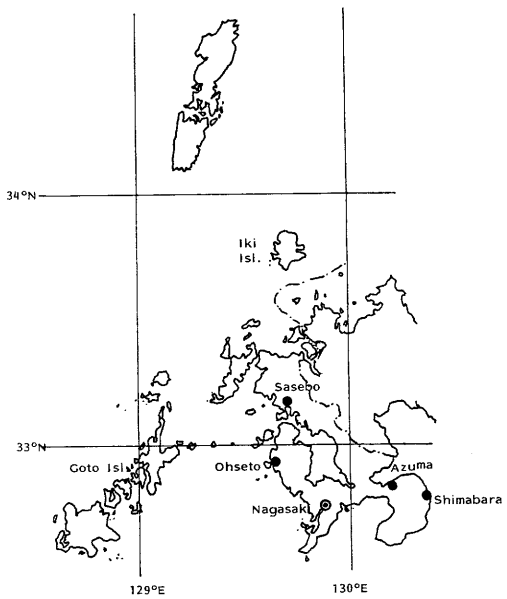


Fig. 1. Sampling sites of bovine sera in Nagasaki Prefecture

*Hemagglutination-inhibition (HI) test*: The method of Clarke and Casals (1958) was used with modification to microtiter system (Sever, 1962).

*Neutralization (N) test*: Microplaque reduction method using BHK21 cells (Hashimoto et al., 1971; Fujita et al., 1975) was modified to use 24-well semimicroplate (FB-16-24-TC, Flow Laboratories, Inc. USA). The Nakayama strain of JE virus was prepared in suckling mouse brains and the supernatant from low speed centrifugation of 10 % homogenate was used as the stock virus. Fifty percent plaque reduction titers were calculated by probit-chart method (Russell et al., 1967) using 10-fold serial dilution of test sera.

**Table 1.** Age of bovines and number of serum specimens tested

| Sampling place | Age (months) in June | Number of serum specimens tested | | | | |
|---|---|---|---|---|---|---|
| | | May | June | July | August | September |
| Iki | 9—13 | | 20 | | 20 | |
| Gotoh | 9—14 | | 20 | 20 | 20 | 20 |
| Saseho | 12 | 21 | 21 | 20 | 16 | 15 |
| Azuma (Shimabara) | 9—14 | | 19 | 20 | 18 | (20)* |
| Ohseto | 1 | | 10 | 9 | 10 | |

*Sampling from Shimabara

*ELISA*: Indirect micromethod of Voller et al. (1976) was used with modifications as described (Igarashi et al., 1981; Bundo et al., 1982). Purified JE vaccine concentrate kindly supplied by the Kannonji Institute, Research Foundation for Microbial Diseases of Osaka University (Takaku et al., 1968) was used as antigen to coat microplate. Test sera were diluted to 1:100 and/or 1:1000 and standard positive serum of known end-point titer was serially diluted with 2-fold steps starting from 1:100 dilution. The diluted sera were reacted with the antigen-coated plate followed by the peroxidase-labelled anti-bovine rabbit IgG (heavy and light chain specific) to measure immunoglobulin ELISA titer (Ig-ELISA). In the case of IgM-ELISA, the plate reacted with test sera were first reacted with anti-bovine rabbit IgG ($\mu$-chain specific), followed by peroxidase labelled anti rabbit IgG goat IgG as described for swine sera (Bundo et al., 1982). Color reaction was developed by *o*-phenylene diamine and $H_2O_2$, and the optical density at 490 nm was recorded by a Micro ELISA Auto Reader (Dynatech) with reference wavelength of 630 nm. ELISA titers of test specimens were calculated by computer system using the standard curve obtained by serial 2-fold dilution of standard serum (Morita et al., 1982).

*Reagents*: Peroxidase-labelled anti-bovine IgG rabbit IgG, anti-bovine IgM rabbit IgG, and peroxidase-labelled anti-rabbit IgG goat IgG were obtained from Cappel Laboratories, Pa. USA. *o*-Phenylenediamine dihydrochloride was the product of Wako Pure Chemicals Co. Osaka.

*Statistical methods*: The methods described by Snedecor (1952) were followed.

## RESULTS

*Correlation between the Ig-ELISA, and HI or N antibody titers of bovine sera aginst JE virus*: Table 2 shows correlation between the Ig-ELISA and HI titers obtained for 318 out of 319 specimens of bovine sera. One of the 319 specimens was not tested by the HI and N tests because of its limitted volume. Specimens showing the HI titers of 10 or more possessed the Ig-ELISA over 200. From this result, we temporarily set the positive limit of Ig-ELISA to 400. Correlation coefficient between the Ig-ELISA and HI titers was 0.49. Table 3 shows correlation between the Ig-ELISA

**Table 2.** Correlation between the Ig-ELISA and HI titers of bovine sera tested against JE virus antigen

| Ig-ELISA | HI | | | | |
|---|---|---|---|---|---|
| | <10 | 10 | 20 | 40 | 80 |
| 50— 99 | 45 | | | | |
| 100— 199 | 77 | | | | |
| 200— 399 | 106 | 2 | | | |
| 400— 799 | 53 | 8 | 1 | 1 | |
| 800—1599 | 7 | 6 | 6 | 2 | |
| 1600—3199 | | | 1 | 2 | 1 |

Total number of specimens: 318

**Table 3.** Correlation between the Ig-ELISA against JE antigen and the N titer against the Nakayama strain of JE virus observed in bovine sera

| Ig-ELISA | N titer | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | <10 | 10 ≀ 19 | 20 ≀ 39 | 40 ≀ 79 | 80 ≀ 159 | 160 ≀ 319 | 320 ≀ 639 | 640 ≀ 1279 | 1280 ≀ 2559 |
| 50— 99 | 31 | 9 | 5 | | 1 | | | | |
| 100— 199 | 56 | 13 | 6 | 2 | | | | | |
| 200— 399 | 62 | 22 | 14 | 10 | | | | | |
| 400— 799 | 15 | 18 | 12 | 12 | 3 | 2 | | 1 | |
| 800—1599 | 2 | 2 | 3 | 7 | 3 | 1 | 1 | 1 | 1 |
| 1600—3199 | | | | | 1 | 2 | 1 | | |

Total number tested: 318

and N titers with correlation coefficient of 0.50. There are many specimens showing low correlation between these 2 parameters especially when their titers are relatively low. Table 4 shows the correlation between the HI and N titers with correlation coefficient of 0.64. Taking the positive limit of antibodies by the HI as 10, by the N as 10, and by the Ig-ELISA as 400, there are positive specimens of 30 by the HI, 153 by the N and 88 by the Ig-ELISA, respectively.

**Table 4.** Correlation between the HI titer against JE virus antigen and the N titer against the Nakayama strain of JE virus as observed in bovine sera

| N titer | HI | | | | |
|---|---|---|---|---|---|
| | <10 | 10 | 20 | 40 | 80 |
| 10 | 164 | 1 | | | |
| 10— 19 | 64 | | | | |
| 20— 39 | 36 | 4 | | | |
| 40— 79 | 21 | 6 | 4 | | |
| 80— 159 | 3 | 2 | 2 | 1 | |
| 160— 319 | | 2 | 1 | 1 | |
| 320— 639 | | | 1 | 1 | 1 |
| 640—1279 | | | | 2 | |
| 1280—2559 | | 1 | | | |

Total number tested: 318

*Changes in the antibody positive rates and the GMT of antibodies during epidemic seasons*: Upper panel of Figure 2 shows changes in the antibody positive rates among bovines during epidemic season in 1981, based on the antibody positive limit described above. In June, none of the 20 bovines in Goto showed antibodies against JE by the HI, N, or Ig-ELISA, while one of the 20 bovines in Iki and 2 of the 10 in Ohseto possessed positive antibodies. On the other hand, in Saseho and Azuma, antibody positive rates in June were relatively high by the N test, 47.6% and 42.1%, respectively. Lower panels in Figure 2 show changes in the GMT during

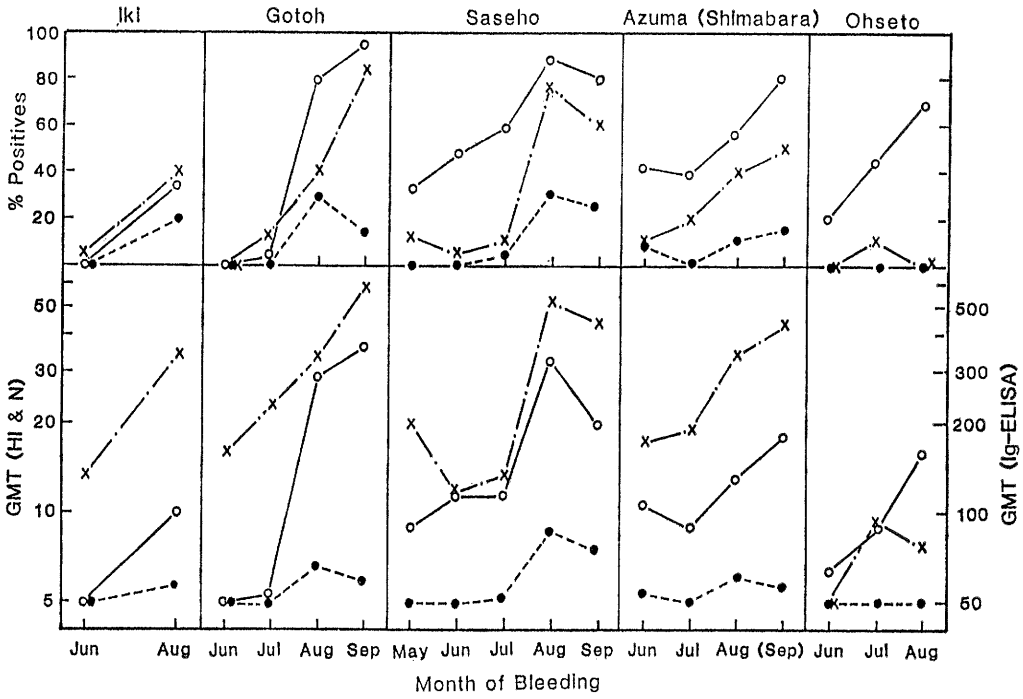


Fig. 2. Changes in anti-JE antibody positive rates and GMTs in bovine sera during epidemic season in Nagasaki, 1981.
Symbols: ● - - - ● HI; ○ —— ○ N; × — • — • × Ig-ELISA.
Titer of positive limit: HI=10; N=10; Ig-ELISA=400.

epidemic season in 1981. The value in June was relatively high in Saseho and Azuma compared with other areas, corresponding to the results of antibody positive rates as described above. However, the GMT of Ig-ELISA in Saseho and Azuma was not significantly high compared with Iki and Gotoh, although the value in Ohseto was lower than other areas.

Compared with these pre-epidemic values, antibody positive rates in August rose up in every area as tested by the N, although the change was not markedly observed by the Ig-ELISA in Ohseto. Also the change by the HI was still lower. Changes in the GMT were also significant either by the N or Ig-ELISA, while the change in HI-GMT was lower. In September, both the antibody positive rates and GMT rose up in Gotoh, however, the values showed slight decrease in Saseho.

*Seroconversion and positive antibody response in bovines during epidemic season in 1981*: Table 5 shows number of bovines showing 4-fold or more rise in their antibodies against JE in August or September compared with the titers measured in June, as determined by the HI, N, and/or Ig-ELISA. Bovine population which showed positive antibody response or seroconversion by all of these 3 serological methods were 6.3% in Au-

gust and 8.8% in September, respectively. While the figures for those showing positive both by the N and ELISA were 6.3% in August and 11.8% in September. The positive rates as detected only by the N in August and September were 19.0% and 32.4%, and by the ELISA 12.7% and 17.6%, respectively.

These figures were summarized into Table 6, which shows the number of bovines with positive antibody response or seroconversion by one of the 3 serological tests used in this study. That is, by the N-test, 32.9 % in August and 52.9 % in September were

**Table 5.** Seroconversion or antibody response in bovines as revealed by the HI, N, and/or Ig-ELISA, in Nagasaki, 1981.

| HI | N | Ig-ELISA | Iki Aug | Gotoh | | Saseho | | Azuma Aug | Ohseto Aug | Aug** | Sep*** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Aug | Sep | Aug | Sep | | | | |
| + | + | + | | 2* | 1 | 3 | 2 | | | 5(6.3) | 3(8.8) |
| + | + | − | | | | | | 1 | | 1(1.3) | |
| + | − | + | | | | 1 | 1 | | | 1(1.3) | 1(2.9) |
| − | + | + | 1 | 2 | 4 | 2 | | | | 5(6.3) | 4(11.8) |
| + | − | − | | | | | 1 | | | | 1(2.9) |
| − | + | − | 4 | 6 | 10 | 1 | 1 | 1 | 3 | 15(19.0) | 11(32.4) |
| − | − | + | 5 | 1 | 2 | 3 | 4 | 1 | | 10(12.7) | 6(17.6) |
| − | − | − | 10 | 9 | 3 | 5 | 5 | 14 | 4 | 42(53.2) | 8(23.5) |
| Total number | | | 20 | 20 | 20 | 15 | 14 | 17 | 7 | 79(100) | 34(100) |

*Figures in the T ble: number of serum specimens
**Sampling in August from Iki, Gotoh, Saseho, Azuma and Ohseto
***Sampling in September from Gotoh and Saseho
Figures in the parentheses: % to total number tested
+: bovine serum specimen showing 4-fold or more rise in antibody titers in August or September as compared with that in June.

**Table 6.** Seroconversion or antibody response among bovines as revealed by the HI, N or Ig-ELISA, in Nagasaki, 1981.

| Serological tests | Iki Aug | Gotoh | | Saseho | | Azuma Aug | Ohseto Aug | Aug* | Sep** |
|---|---|---|---|---|---|---|---|---|---|
| | | Aug | Sep | Aug | Sep | | | | |
| HI | | 2 | 1 | 4 | 4 | 1 | | 7(8.9) | 5(14.7) |
| N | 5 | 10 | 15 | 6 | 3 | 2 | 3 | 26(32.9) | 18(52.9) |
| Ig-ELISA | 6 | 5 | 7 | 9 | 7 | 1 | | 21(26.6) | 14(41.2) |
| Total number | 20 | 20 | 20 | 15 | 14 | 17 | 7 | 79(100) | 34(100) |

*Sampling from Iki, Gotoh, Saseho, Azuma, Ohseto.
**Sampling from Gotoh, Saseho
Figures represent number of bovines showing 4-fold or more rise in antibodies as revealed by the HI, N, or Ig-ELISA.
Figures in the parentheses: % to total number tested.

positive; by the HI, 8.9 % in August and 14.7 % in September; and by the Ig-ELISA, 26.6 % in August and 41.2 % in September, respectively.

*Measurement of IgM-ELISA titers*: Tests were performed as described in the Materials and Methods, however, the titers of IgM-ELISA observed for bovine sera were relatively low. The values obtained for each specimen were summarized in Table 7. It seems that the IgM-ELISA titer is higher in Saseho and Azuma where the Ig-ELISA titer is also high. The result might represent some booster phenomenon, however, the interpretation should wait for further critical examinations.

**Table 7.** IgM-ELISA titers in bovine sera in Nagasaki, 1981

| Sampling place | Sampling time | | | | |
|---|---|---|---|---|---|
| | May | June | July | August | September |
| Iki | | 84(50-202) | | 90(50-167) | |
| Gotoh | | 61(50-104) | 85(50-226) | 84(50-254) | 87(50-382) |
| Saseho | 104(50-225) | 102(50-277) | 201(50-419) | 235(50-351) | 178(50-400) |
| Azuma | | 174(50-273) | 182(50-362) | 193(110-376) | 221(50- 00)** |
| Ohseto | | 50(50-50) | 83(50-400) | 94(50-227) | |

*Figures represent GMT of IgM-ELISA, with the lowest and the highest titers in the parentheses. Figure 50 means the titer less than 100.

**sampling in September was done in Shimabara

## DISCUSSION

Difference was observed in the antibody positive rates in May or June according to the sampling place, especially low GMT value in Ohseto was remarkable. The latter result may be due to the fact that the bovines in Oseto were newly introduced from Hokkaido, JE-nonendemic area, at the age of 1 month old. On the other hand bovines in other areas may have been exposed to JE virus infection in the previous year. Positive antibody rate, as well as GMT value were found to increase in August or September compared with those in June, indicating quite widespread exposure of bovine population to JE virus in the epidemic season in Nagasaki, 1981. The antibody positive rate in August or September were 80-95 % as detected by the N, the value is higher than those reported by Miyata (1981) for bovine serum specimens in Kagoshima, another Prefecture in Kyushu island. Mitamura et al., (1938) observed the antibody positive rate of bovines in Kobe as 48.4 %, in Tokyo and Yokohama as 88.1 %, and in Hokkaido as 0 %, when they determined by the N-test. Yamada et al. (1972) reported the antibody prevalence of bovines between 0-13 % in Hokkaido.

Compared with the results of bovines, 100 % of swine population was found to possess antibodies against JE virus on the 25th of August either by the HI and ELISA (Igarashi et al., 1981; Bundo et al., 1982). Also the antibody titers of swine were

much higher than those in bovines. These results agree with observations by Matsuo et al. (1968) and Otsuka et al. (1969) showing that the antibody titers in bovines as well as the sensitivity of bovines against JE virus is lower than swine.

From our observations, 11 % of the serum specimens showed negative N-titer with fairly high Ig-ELISA over 400. The result might be due to the fact that the Nakayama strain was used in the N-test. Another possibility is that these bovines produced antibodies directed to the antigenic site(s) responsible for the ELISA but not for the N-test. Such antigenic site(s) were described by Kimura et al. (1983) using monoclonal antibodies against JE virus. On the other hand, 20.3 % of August specimens and 32.4 % of September specimens were found to show 4-fold or more rise by the N-test without showing positive antibody response by the ELISA. These results may partly be due to the high background in the ELISA, resulting in the negative antibody response. Nonspecific reactions in the ELISA have been described by Miyata (1981). Another possibility is similar to the above mentioned specificity of multiple antigenic sites on the virions, indicating the production of N- antibodies without showing high levels of ELISA-specific antibodies.

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# 1981年長崎県における牛血清の日本脳炎ウイルスに対する抗体価について

分藤桂子，森田公一，五十嵐　章（長崎大学熱帯医学研究所ウイルス学部門）
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1981年夏，長崎県下の5地域より得られた定地牛血清の日本脳炎（JE）ウイルスに対する抗体価を，血球凝集抑制反応（HI），中和反応（N），及び免疫酵素測定法（ELISA）により測定した．全地域において，流行期に抗体保有率と幾可平均値の上昇がみられ，牛の JE ウイルスの感染は広い範囲に及んでいることが証明された．JE ウイルス感染の検出率は，Nで最も高く，次いで ELISA，HI の順であった．この抗体価と抗体保有率をブタと比較してみると，牛はブタに比べて JE に対して感受性が低いと思われる．

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