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ANTIBODY ASSAY FOR JAPANESE ENCEPHALITIS VIRUS IN BOVINE SERUM BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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Enzyme-linked immunosorbent assay (ELISA) was applied for seroepidemiological study of Japanese encephalitis (JE) using bovine sera. ELISA antibody titers were four fold higher than neutralizing antibody titers. Minimum titer of positive ELISA antibody was set at a serum dilution of 1:40 to eliminate the non-specific reaction against normal mouse brain components in a test antigen. Antibody prevalence rates against JE virus in Kagoshima, an endemic area, were 26.0% in ELISA and 23.5% in the neutralization test. Meanwhile, sera from Hokkaido, a JE free area, had a prevalence rate of 4.3% by ELISA. Cross-reaction in ELISA between JE and Negishi virus was seen in bovine sera from Kagoshima and also in immune rabbit sera.

INTRODUCTION

ELISA was developed in a study to detect an antigen in tissue by NAKANE and PIERCE¹¹⁾ in 1966. ENGVALL and PERLMANN⁴⁾ in 1971 reported a quantitative analysis of rabbit IgG by using enzyme labeled anti-rabbit IgG. Since then, ELISA has been applied not only in the quantitation of antigen and antibody^{3,4,5)} but also in the diagnoses of infectious diseases^{12,13,14,15,16)} because of its sensitivity, simplicity, rapidity and economy.

Epidemiological studies of JE for the forecasting of epidemics have mainly concentrated on the surveillance of JE virus antibody in swine populations.^{8,9)} Since swine are usually sent to the slaughterhouse around 6 months of age, antibody prevalence reveals only newly acquired infection. On the other hand, cattle are also susceptible to many arboviruses, and they retain them longer than do swine. Thus cattle are suitable for a retrospective study by age distribution of antibodies against JE and other arbovirus infections.

This study describes the use of ELISA instead of HI and neutralization test for sero-epidemiological survey of JE using bovine sera from an endemic and non-endemic area of JE.

MATERIALS AND METHODS

Bovine sera: The sera were collected from 486 animals in Abashiri and Soya districts of Hokkaido in 1978 and from 204 animals in Kagoshima prefecture in 1971. These were heat inactivated at 56°C for 30 minutes before use.

Antisera: Anti-JE serum was prepared in a rabbit by 6 subcutaneous injections of purified JaGAR-01 strain of JE, which was supplied by Dr. H. NAKAMURA of Nippon Institute for Biological Science, Tokyo, Japan. Anti-Negishi serum was made in a rabbit by 6 intramuscular injections of an equal mixture of Freund's complete adjuvant and Negishi virus antigen as two per cent brain suspension of infected suckling mice. Anti-bovine IgG serum was prepared in a rabbit by three repeated intramuscular injections of an equal mixture of Freund's complete adjuvant and 1 mg of purified bovine IgG fraction. The titer of the anti-bovine IgG serum was 1:400 in a double gel immunodiffusion test. Anti-rabbit IgG serum was prepared in a sheep using purified rabbit IgG fraction by the same immunization method as that of the anti-bovine serum preparation.

Viruses: JaGAR-01 strain of JE virus and Negishi virus of tick borne encephalitis virus complex were obtained from Dr. A. OYA, National Institute of Health, Tokyo, Japan. The viruses were inoculated intracerebrally into suckling mice. Two per cent suspension of the infected suckling mouse brains was prepared in phosphate buffered saline (PBS) containing two per cent of fetal calf serum (FCS) and centrifuged at 10,000 rpm for 20 minutes. The supernatant was stored at -85°C and used as a virus stock.

Sucrose acetone extracted (SA) antigen: SA antigens were prepared from the brains of infected suckling mice by the sucrose acetone extraction method of CLARKE and CASALS.²⁾

Tissue culture: BHK-21 were grown on tissue culture plates of 5.5 cm in a diameter (Falcon, USA) with Eagle's MEM containing 10% calf serum at 37°C in a 5% CO₂ incubator.

Fifty per cent plaque neutralization test: The test was performed according to the method described in reference.⁷⁾ Briefly, a virus suspension containing approximately 200 plaque forming units (PFU)/0.2 ml was mixed with an equal volume of serum diluted in two-fold steps and incubated at 37°C for 90 minutes for virus neutralization. Two tenth ml of the mixture was inoculated onto the monolayer cell sheet and adsorbed at 37°C for 90 minutes. The plate was covered by carboxy methyl-cellulose (CMC) overlay medium and incubated for 3 days at 37°C in a CO₂ incubator. The plate was then, fixed by Müller's solution containing 5% formalin and stained with 0.1% trypan blue solution. The neutralizing antibody titer was expressed as the serum dilution which reduced more than 50% of the plaque counts of the control plate without the test serum.

Acetone treatment of serum: Test serum (0.2 ml) was mixed with four ml of cold

acetone. The mixture was shaken well and centrifuged at 1,500 rpm for 5 minutes, and the precipitate was resuspended in four ml of cold acetone. After three successive treatments, the precipitate was dried under negative pressure and dissolved in 1 ml of PBS as a serum 1:5 dilution.

ELISA: Anti-bovine IgG and anti-rabbit IgG were conjugated with horseradish peroxidase (HRP) (type VI, Rs=303, Sigma, USA) according to the method of NAKANE and KAWAOI.⁸⁾ One tenth ml of SA antigen diluted in 0.06 M sodium carbonate buffer was absorbed on the wells of a polystyrene microplate (96 U, PS. SH, Nunc, Denmark) by the method of FORGHANI et al.⁶⁾ at 4°C for 12 hours. Eight units of SA antigen and 4 units of conjugate were used for the test according to the box titration of SA antigen, conjugate and anti-viral antiserum. The substrate used was 4 mM of 5-amino-salcylate (MW: 204.55, Tokyo Kasei, Japan) dissolved in boiling distilled water. One per cent H₂O₂ was added to the substrate solution, and pH of the solution was adjusted at 6.0 with 1 N NaOH. The washing solution consisted of PBS containing 0.05% polyoxyethylene sorbitan (Tween-20) (pH 7.4). Wells coated with antigen were washed once with the washing solution and filled with 0.025 ml of PBS. The serum was diluted serially using a loop (0.025 ml) on the transfer plate (Falcon, USA) and transferred to the antigen plate. After incubation at room temperature for 40 minutes, the serum was removed from the wells and washed three times by the washing solution. The wells were filled with 0.025 ml of conjugate (4 units) and incubated at room temperature for 40 minutes. After removing the conjugate, the wells were washed three times, added to the 0.1 ml of substrate solution and incubated with a cover at room temperature overnight. The coloring reaction was determined by O. D. value at 400 nm or by viewing with the naked eye. Results were obtained only from the plates showing no reaction in the wells of known negative sera or from these positive reaction in the wells of known positive sera.

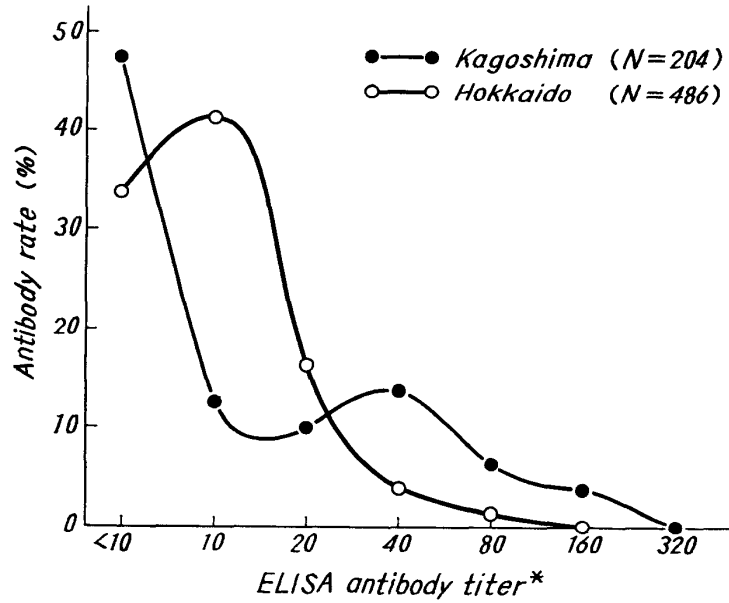
RESULTS

Distribution of ELISA antibody titers and comparison of the titers in ELISA and neutralization test

Distribution of ELISA antibody titers against JaGAR-01 strain was examined in bovine sera from Kagoshima prefecture, which is known as an endemic area of JE and from Hokkaido a non-endemic area. The results are shown in Fig. 1. ELISA titers of sera from Kagoshima distributed with two peaks at less than 1:10 and at 1:40. ELISA titers of sera from Hokkaido, however, fell into one peak at 1:10.

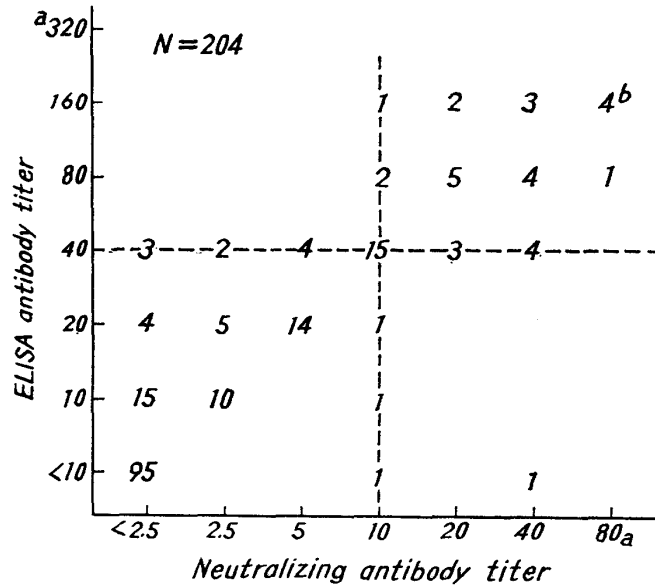
Consequently, the ELISA antibody and neutralizing antibody titers against JaGAR-01 strain in the sera from Kagoshima were compared to check the sensitivity of reaction in ELISA (Fig. 2). High correlation was seen in antibody titers between ELISA and the neutralization test. In this ELISA system, antibody titers were almost four

FIGURE 1 *Distribution of ELISA antibody titers against JE virus in Kagoshima and Hokkaido sera*



* Reciprocal of serum dilution

FIGURE 2 *A comparison of ELISA and neutralizing antibody titers against JE virus in Kagoshima sera*



a : reciprocal of serum dilution

b : number of sera

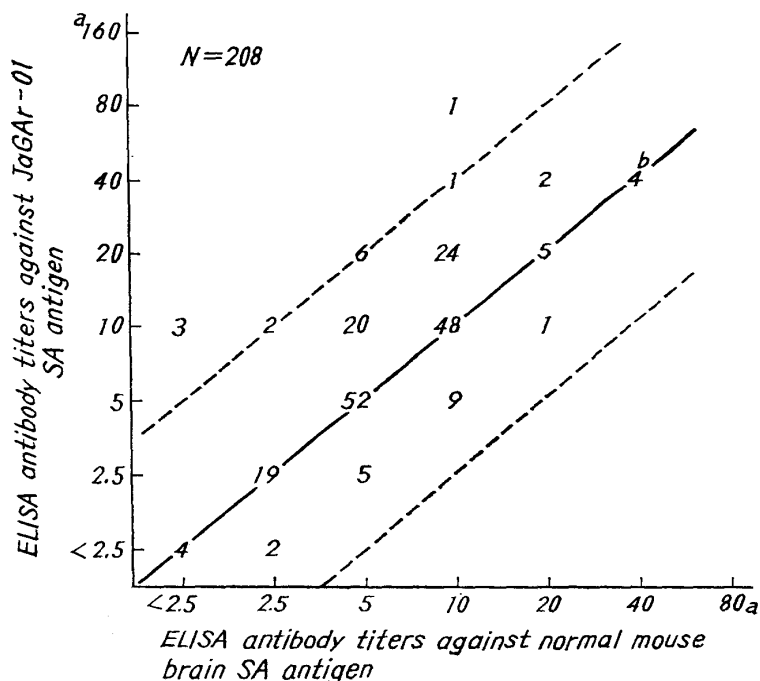
fold higher than those in the neutralization test. Of 48 sera with neutralizing antibody titers of 1:10 or more, 45 sera (93.7%) had ELISA antibody titers of 1:40 or more. Only 9 out of the 159 sera with neutralizing antibody titers of less than 1:10 (5.7%) had ELISA antibody titers of 1:40. ELISA antibody titer of 1:40 was equivalent to the neutralizing antibody titer of 1:10 which were read usually as minimum titers for positive antibody in the neutralization of arboviruses. Therefore, the minimum antibody titer for positive ELISA was set at 1:40 in this system with bovine sera.

Elimination of non-specific reaction in ELISA

In CF and HI tests of arboviruses acetone treatment of sera was required for elimination of non-specific reaction. The sera were examined for ELISA antibody titers against JE virus before and after acetone treatment. No obvious differences were seen, however, between them (result not shown).

To clarify the involvement of non-specific reaction due to mouse brain component contained as a contaminant in the viral antigen, the sera from northern Hokkaido, known as a JE free area, were compared for ELISA titers both against JaGAR-01 SA and normal mouse brain SA antigens (Fig. 3). ELISA antibody titers against both

FIGURE 3 A comparison of ELISA antibody titers against JE SA antigen and normal mouse brain SA antigen in Hokkaido sera



a : reciprocal of serum dilution
 b : number of sera

antigens were within two fold difference in 195 sera out of 208 sera (93.8%), and they were distributed with a single peak at 1:5 or 1:10. Therefore, most of the titers against both antigens were considered to be against normal mouse brain components. Eight sera had ELISA antibody titers against JaGAR-01 SA antigen equal to or more than 1:40, and 4 out of 8 sera also had titers of 1:40 against normal mouse brain SA antigen. Thus, very few sera (1.9%) reacting against normal mouse brain SA antigen may be considered as false positive when the minimum positive titer is set at a dilution of 1:40.

Cross-reaction between JE and Negishi virus among Flavivirus using their antisera in ELISA

ELISA and neutralizing antibody titers were examined in rabbit hyper-immune sera against JaGAR-01 strain of JE virus and Negishi virus of tick borne encephalitis complex (Table 1). Eight fold difference of ELISA antibody titers was observed in JaGAR-01 antiserum against two SA antigens. ELISA titer of rabbit anti-Negishi serum against homologous Negishi SA antigen was two fold higher than that against JaGAR-01 SA antigen. ELISA titer of the serum against normal mouse brain SA antigen was low, and thus the cross reactions of the serum against the two viruses were not considered to be due to the reaction against normal mouse brain SA antigen. Neutralizing antibody titers were low in a heterologous combination of immune sera vs. viruses in contrast with ELISA titers.

Antibody rates of bovine sera against JE and Negishi viruses from Kagoshima prefecture and Hokkaido were compared in both ELISA and the neutralization test (Table 2). Antibody rates of JaGAR-01 in Kagoshima sera were almost the same in ELISA and in the neutralization test. ELISA antibody rate of JaGAR-01 was low (4.3%) in Hokkaido. The prevalence rate of Negishi antibody using ELISA was 19.6% in the sera from Kagoshima, which was higher than 9.9% as compared to the sera from Hokkaido. Higher ELISA positive rate against Negishi virus in Kagoshima may be due to cross-reaction of Negishi virus against the antibodies produced by JE virus infection.

TABLE 1 *Cross-reaction between JE and Negishi viruses in the rabbit immune sera*

SA ANTIGEN	ELISA ANTIBODY TITER RABBIT IMMUNE SERA		NEUTRALIZING ANTIBODY TITER RABBIT IMMUNE SERA	
	JaGAR-01	Negishi	JaGAR-01	Negishi
JaGAR-01	640*	5,120	320*	<10
Negishi	80	10,240*	<10	5,120*
Normal mouse brain	10	640	NT	NT ^a

*: reaction between homologous combination

a: not tested

TABLE 2 *Prevalence rates of antibody to JE and Negishi viruses in bovine sera from Kagoshima and Hokkaido in both ELISA and Neutralization test*

ANTIGEN	ASSAY	ANTIBODY PREVALENCE RATES IN	
		Kagoshima	Hokkaido
JE (JaGAR-01)	ELISA	53/204 ^a (26.0) ^b	21/486 (4.3)
	Neutralization	48/204 ^c (23.5)	NT ^d
Negishi	ELISA	40/204 (19.6)	48/486 (9.9)
	Neutralization	8/204 (3.9)	NT

a : number of positive sera (higher than 1:40)/total number of sera

b : per cent of positive sera

c : number of positive sera (higher than 1:10)/total number of sera

d : not tested

DISCUSSION

Since establishment of the HA system of arboviruses by CASALS and BROWN,¹⁾ the HI test has been commonly used because of its simplicity and reliability for serological diagnosis of arbovirus infection. However, the system requires acetone treatment of sera or adsorption of sera by caolin to remove lipids as HI inhibitors²⁾. In this study no prior acetone treatment of the sera was necessary in ELISA because acetone-soluble non-specific substances were not involved in the assay. Moreover, although the neutralization test is highly specific in the serological differentiation of arboviruses, it is laborious and time consuming. As seen in Fig. 1, ELISA antibody titers were almost four fold higher than neutralizing antibody titers. From the comparative results of two tests in bovine sera from Kagoshima where JE was endemic, very similar prevalence rates were obtained when the minimum titers of 1:40 for ELISA and 1:10 for neutralization were set as positive antibodies. If the minimum titers for positive ELISA antibody were set at 1:80, most of the sera with low neutralizing antibody titers (1:10) were judged as JE antibody negative. On the other hand, only a few sera (1.9%) from northern Hokkaido known as a JE free area showed a titer of 1:40 or more against JaGAR-01 SA antigen. But ELISA titers lower than 1:40 were observed mostly to be non-specific reactors against mouse brain components in SA antigen. Therefore, we recommended that further purified viral antigen should be used to eliminate the false positive reaction in ELISA.

Higher cross-reaction between JE and Negishi viruses was observed in ELISA than in the neutralization test using hyperimmune sera. This might have been due to the presence of a common antigen in the flaviviruses, because ELISA titers of the sera were lower against the normal mouse brain antigen than against the viral antigens. Generally, ELISA can detect the antibodies against all of the viral antigens include a common antigen which may be an inner component of flaviviruses. In the neutralization test, however, only antibodies against the surface or membrane antigen which is highly specific in antigenicity are detectable.

The prevalence rate of Negishi virus antibody in the bovine sera of Kagoshima by ELISA was higher than that in the neutralization test. These results in ELISA may have been due to a cross-reaction against the antibodies produced by JE virus or another flavivirus infection, if any. The availability of the ELISA for sero-epidemiological survey was reconfirmed from the great difference in prevalence rates of JE virus infection between Kagoshima as an endemic area and Hokkaido as a non-endemic area.

Several of the positive sera judged by the neutralization test might have been judged as negative by ELISA because of the difference in involvement of immunoglobulin classes between both tests. The neutralization test may be mediated by IgA, IgG and IgM antibodies. However, in ELISA only anti-bovine IgG conjugate was used for detection of specific viral antibodies; therefore, ELISA could not detect IgA and IgM antibodies in this study although anti-bovine IgG (H and L chain) was not mono-specific to the γ chain of IgG. Further studies on the use of ELISA using anti-bovine IgM conjugate are necessary for detection of IgM antibody as a marker of early infection of JE virus.

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