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LABELED AVIDIN-BIOTIN ENZYME-LINKED[1] IMMUNOSORBENT ASSAY (LAB-ELISA) FOR DETECTION OF JAPANESE ENCEPHALITIS ANTIBODY IN SWINE SERA

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Labeled avidin-biotin (LAB) enzyme-linked immunosorbent assay (ELISA) was applied to detect Japanese encephalitis (JE) antibody in swine sera. Purified JaGAr-01 vaccine of JE, biotin labeled anti-swine Ig or IgM rabbit IgG conjugate and horseradish peroxidase (HRP) were employed in the assay. The μ chain specific anti-IgM conjugate could detect only IgM antibody, but the anti-Ig conjugate could detect both IgM and IgG antibodibes to JE.

When 2-2′-azino di-[3-ethylbenzthiazoline sulphonic acid] (ABTS) was used as a substrate to HRP, sensitivity of LAB-ELISA was essentially the same as that of ordinary ELISA, but approximately 20 times higher than that of hemagglutination inhibition (HI) test. LAB-ELISA titers with ABTS were approximately 5 times higher than those with 5-amino salcylic acid (5-AS). However, non-specific reactions of sera were about 4 times lower in LAB-ELISA than in ordinary ELISA.

Key word: LAB-ELISA, avidin-biotin, Japanese encephalitis, swine

INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) has been applied widely as an economic, sensitive and non-hazardous serodiagnostic method for arbovirus infections as well as hemagglutination inhibition (HI), complement fixation and neutralization tests.[2,3,7,8,11,12,13]However, ELISA techniques for antibody detection vary in the combination of coating antigens, enzymes and substrates. OHKUBO *et al.*[13] reported the successful titration of JE antibody in swine sera by ELISA using the sucrose-aceton extracted (SA) antigen, peroxidase labeled immunoglobulin and 5-amino salcylic acid (5-AS) substrate. However, a high level of non-specific reaction remained even though purified JE antigen was used.

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Recently avidin-biotin system have been introduced in ELISA by GUESDON *et al.*[6] Avidin is a glycoprotein found in egg white, and it binds specifically to biotin.[5] It can be conjugated to enzymes such as horseradish peroxidase (HRP), and biotin can be coupled with free amino groups in proteins such as IgG.[6] Both procedures can be accomplished without any intricate technique, making the avidin-biotin interaction a convenient system in immuno-enzyme assay. This interaction has been applied already in ELISA for the identification of immunoglobulins and the detection of antigens,[6,9,15] but it has not been widely explored yet as a useful technique in serodiagnosis of infectious diseases.

In this paper, avidin-biotin system was incorporated in ELISA for the detection of JE antibody in swine sera using commercially available reagents. The sensitivity of the antibody detection and the level of non-specific reaction in the labeled avidin-biotin ELISA (LAB-ELISA) were compared with those in ordinary ELISA and HI test. Furthermore, two different substrates, 5-AS and ABTS, were tested in order to determine the optimal ELISA system for sero-diagnosis.

MATERIALS and METHODS

Antigen

Purified JE vaccine (total-N: 96.6 μg/ml), JaGAr-01 strain, was kindly supplied by Dr. H. TAKAGI, Biken, Osaka, and was used as an antigen in this study. Procedure for purification of JE vaccine was described elsewhere.[16]

Sera

Anti-swine Ig serum was prepared in a rabbit by 3 successive inoculations of an equal mixture of Freund's complete adjuvant and swine IgG (1 mg, whole molecule), and fractionated through a Sephadex G-200 column. The anti-swine IgM made in the rabbit (μ-chain specific) was purchased from Miles Laboratories, INC., U. S. A.

A SPF swine was inoculated subcutaneously with 10^5 PFU of JE virus, JaGAr-01 strain. Sera were obtained from the swine prior to inoculation and at intervals after the virus inoculation. Sera were also collected from swine in Shizuoka prefecture, which was noted as an endemic area of JE, in September of 1980, and in northern Hokkaido, which is a non-endemic area, in August of 1980. All of the sera were inactivated at 56°C for 30 minutes and stored at −40°C until used.

Biotinylation of immunoglobulins

Biotin-labeled anti-swine Ig or IgM rabbit IgG (biotin-anti-Ig or anti-IgM) were prepared as described by Guesdon *et al.*[6] The rabbit IgG of the antiserum was purified by chromatography on a DEAE cellulose (Whatman DE 52) column. The IgG fraction was dialyzed in 3 changes of 0.1 M $NaHCO_3$ at 4°C. After dialysis, 1 ml of IgG (1 mg/ml) was mixed with 50 μl of biotin-N-hydroxy succinimide ester (BHSE) (E-Y, LABS, INC., U. S. A., 1 mg/ml in dimethyl sulfoxide) in a test tube. The mixture was incubated at room temperature for 4 hours, then transferred to a

cellulose tube and dialyzed at 4°C against 4 changes of PBS to remove any free biotin ester and dimethyl sulfoxide. Finally, the biotin-labeled antibody was distributed into small vials, stored at −80°C and used as a working solution of biotin-anti-Ig or biotin-anti-IgM.

Horseradish peroxidase (HRP) labeled avidin

HRP labeled avidin (HRP-avidin) was purchased from E-Y, LABS, INC., U. S. A., and also distributed into small vials and stored at −80°C until used.

HRP-labeled immunoglobulins

To prepare the conjugate for ordinary ELISA, anti-swine Ig or IgM (μ chain specific) was conjugated with horseradish peroxidase (type VI, Sigma, U. S. A) by the one-step glutaraldehyde procedure, as previously described.[1] The conjugate (HRP-anti-Ig or HRP-anti-IgM) was also distributed into small vials and stored at −80°C until used.

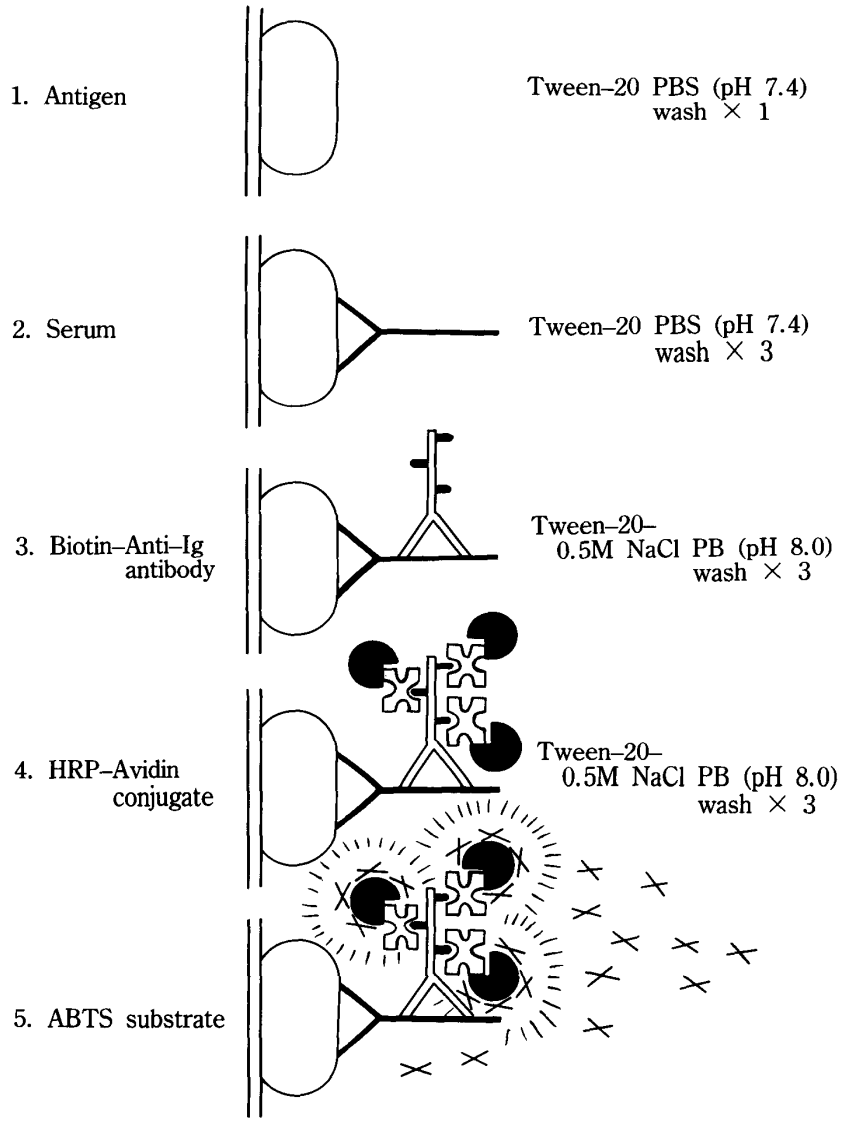
Substrate

Two kinds of substrates were used in this study. Four mM solution of 5-AS (Tokyo Kasei) was prepared in boiling double-distilled water. The solution was cooled, and the pH was adjusted to 6.0 by adding IN NaOH. One ml of 1% H_2O_2 was added to 20 ml of the solution and used as a 5-AS substrate. Forty mM solution of ABTS (Sigma, U. S. A.) was prepared in double-distilled water. Fifty μl of the solution were mixed with 10 ml of 0.05M citrate buffer, pH 4.0, and 40 μl of 1% H_2O_2, and the mixture was used as an ABTS substrate.[10]

Procedure of LAB-ELISA

The procedure was illustrated in Fig. 1. JE antigen was dissolved in the 0.05 M carbonate buffer, pH 9.6, at a concentration of 8 units, and 100 μl of the diluted antigen were delivered into the wells of a U-shaped microtiter plate (96U. PS. SH. Nunc, Denmark). The antigen was fixed on the wells by incubation at 37°C for 3 hours. The wells were washed with the solution of phosphate buffered saline (PBS) (pH 7.4) containing 0.05% Tween 20 (Tween-20 PBS). The swine serum was diluted in the solution of PBS containing 0.1% Tween 80 (Tween-80 PBS). Fifty μl of the diluted serum were delivered into the wells of the plate coated with the antigen. The plate was incubated at 37°C for 40 min and then washed 3 times with Tween-20 PBS. Biotin-anti-Ig or biotin-anti-IgM was diluted in Tween-80 PBS at a concentration of 4 units, and 50 μl of the solution were added to each well. The plate was incubated at 37°C for 40 min and then washed 3 times with 0.05M phosphate buffer, pH 8.0, containing 0.1% Tween 20 and 0.5M NaCl (Tween-20 0.5M NaCl PB). HRP-avidin was prepared at a concentration of 8 units in Tween-20 0.5M NaCl PB and 50 μl of the HRP-avidin were distributed into the wells. The plate was incubated at 37°C for 40 min and washed 3 times with Tween-20 0.5M NaCl PB. One hundred μl of 5-AS substrate or ABTS substrate were next poured into the wells. The plate was incubated at 37°C for 60 min. Coloring reaction of 5-AS was ceased by adding 100 μl of 1N NaOH. The degree of reaction was read on the spectrophoto-

FIGURE 1 *Flow diagram of Labeled Avidin-Biotin (LAB) ELISA.*



meter for microplate (Corona MTP-12) at wave lengths of 492 nm for 5-AS substrate and 405nm for ABTS substrate.

Procedure of ordinary ELISA

The procedure was followed by the method reported by OHKUBO *et al.*[13] Fixation of antigen, binding of JE antibody to antigen plate and reaction with substrate were essentially

the same as those of LAB-ELISA. Fifty μl of HRP-anti-Ig or HRP-anti-IgM conjugate were delivered into the wells after binding of JE antibody, and the plate was incubated at 37°C for 40 min. The plate was washed 3 times with Tween-20 PBS, followed by addition of substrate solution.

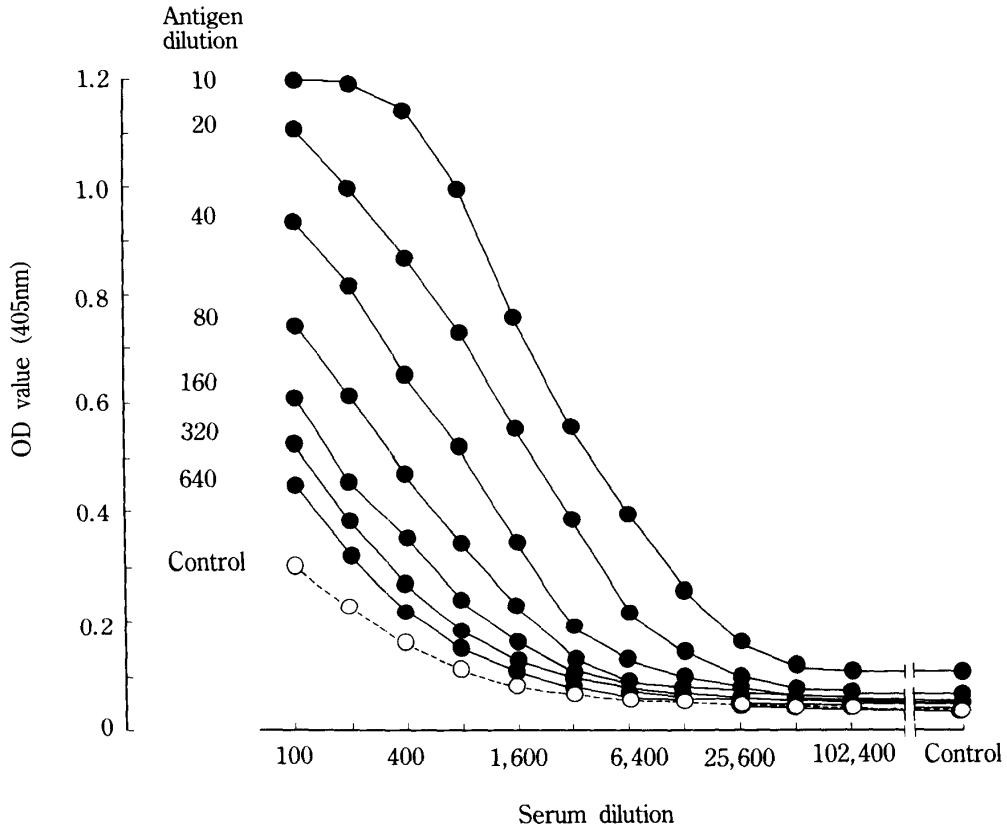
Procedure of HI test

The procedure was followed by the method of Clarke and Casals.[4] Procedure for 2-ME treatment of sera was described elsewhere.[14]

Results

To standardize the reagents in LAB-ELISA with ABTS, the antigen and the antibody concentrations were determined at first by checkerboard titration (fig. 2). JE antigen was diluted serially, fixed on the wells and reacted with a serially diluted antiserum obtained from an experimentally infected swine, No. 1, 11 weeks after the

FIGURE 2 *Checkerboard titration between JE antigen and antiserum in LAB-ELISA*



The antigen was used with JaGAr-01 vaccine. JE antiserum was from an experimentally infected swine, No. 1, 11 weeks after virus inoculation. Biotin-anti-Ig was used at a dilution of 1 : 400 and HRP – avidin at 1 : 1,600. ABTS was used as a substrate.

virus inoculation. Biotin-anti-Ig was used at a concentration of 1: 400 (8 units) and HRP-avidin conjugate at a concentration of 1 : 1,600 (16 units). At a certain concentration of antiserum, OD values decreased in accord with the degree of dilutions of antigen. Furthermore, at each different concentration of antigen, OD values decreased linearly in accord with the degree of dilutions of antiserum, except for the highest concentration of antigen (1 : 10), at which the OD values reached a plateau with antiserum dilutions of 1 : 400 or lower. Very minor reactions were observed in the control wells without serum and antigen. In the control wells without antigen but with an antiserum of 1 : 100 to 1 : 400, OD values were slightly higher than those with the antigen, but the values were still lower than those containing the antigen of the lowest dilution (1 : 640).

The cut-off point was set at the sum of the average OD values of the control wells without serum and antigen and an additional factor of 0.1. According to the above criteria, when the antiserum was used at a dilution of 1 : 800, the end point titer of antigen was determined as 1 : 160. End point titers of other reagents were determined, as in the case of antigen, by using two kinds of substrates, ABTS and 5-AS, both in LAB-ELISA and ordinary ELISA (tab. 1).

ABTS substrate gave a markedly sensitive reaction as compared with 5-AS substrate in both ELISAs. End point titers of each reagent with ABTS were expressed about 4 times higher than those with 5-AS. There were no essential differences in the end point titers of the reagents between LAB-ELISA and ordinary ELISA. One mg per ml of IgG fraction of anti-swine Ig was conjugated with biotin to prepare

TABLE 1 *End point titers of reagents in LAB-ELISA and ordinary ELISA with 5-AS and ABTS*

PROCEDURE	REAGENT	END POINT TITER* OF REAGENT WITH SUBSTRATE		UNIT OF REAGENT USED
		5-AS	ABTS	
LAB-ELISA (Ig)	Antigen	40	160	8
	Antiserum (0773)	1,280	6,400	4
	Biotin-anti-Ig	800	3,200	4
	HRP-avidin	6,400	25,600	8
Ordinary ELISA (Ig)	Antigen	40	160	8
	Antiserum (0773)	1,280	6,400	4
	HRP-anti-Ig	3,200	12,800	4

* Titer was expressed as the reciprocal of a maximum dilution of reagent with positive reaction.

biotin-anti Ig conjugate. For preparation of HRP-anti-Ig conjugate, 5 mg/ml of IgG fraction of the same anti-swine Ig was employed. Thus, IgG concentration of anti-Ig conjugate for LAB-ELISA was one fifth of that for ordinary ELISA. Although end point titer of biotin-anti-Ig conjugate was 4 times lower than that of HRP-anti-Ig conjugate both conjugates of same units contained almost equal concentration of IgG. The units of reagents shown in Table 1 were used in subsequent studies.

Specificities of biotin-labeled anti-IgM and anti-Ig conjugates were examined in LAB-ELISA with ABTS (tab. 2). Serum from No. 1, examined at 9 days after inoculation of JaGAr01 strain, had an equal titer of JE antibody against both biotin-anti-IgM and biotin-anti-Ig conjugates. The serum was applied onto a Sephadex G-200 column, and the peak fractions of IgM and IgG of the serum were tested to the conjugates. Anti-IgM conjugate gave an antibody titer of 1 : 200 against IgM fraction and no reaction against IgG fraction. The same results were also obtained in sera from swine No. 1 at 11 weeks after infection, and swine No. 0778, which was naturally infected. On the other hand, the anti-Ig conjugate gave an antibody titer of 1 : 100 against IgM fraction and a titer of 1 : 25 against IgG fraction in the serum from swine No.1 at 9 days after the virus inoculation. However, the anti-Ig conjugate against IgM fraction gave a titer similar to that of anti-IgM conjugate against IgM fraction. The same results were also obtained in sera from swine No. 1 at 11 weeks after the infection and from swine No. 0778. These results showed that the anti-Ig conjugate reacted to both IgM and IgG antibodies, and that the anti-IgM conjugate reacted only to IgM antibodies.

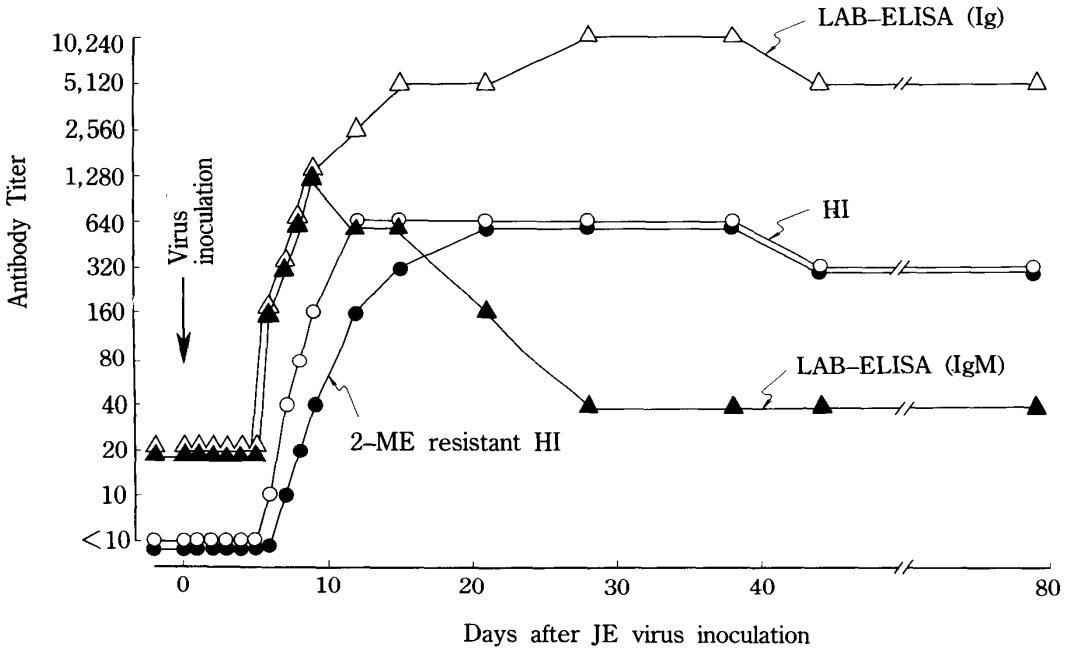
TABLE 2 *Specificity of anti-Ig and anti-IgM conjugates examined by reacting the conjugates against IgM and IgG fractions in LAB-ELISA with ABTS*

SWINE SERUM	TITER WITH CONJUGATE		PEAK FRACTION	TITER IN PEAK FRACTION WITH CONJUGATE	
	anti-IgM	anti-Ig		anti-IgM	anti-Ig
No. 1, 9 days*	1,600**	1,600	IgM	200	100
			IgG	<1	25
No. 1, 11 weeks*	50	6,400	IgM	12	12
			IgG	<1	800
No. 0778	6,400	12,800	IgM	800	400
			IgG	<1	800

* Serum was collected from swine No. 1, 9 days (or 11 weeks) after JE virus inoculation.

** Titer was expressed as the reciprocal of a maximum dilution of serum with positive reaction.

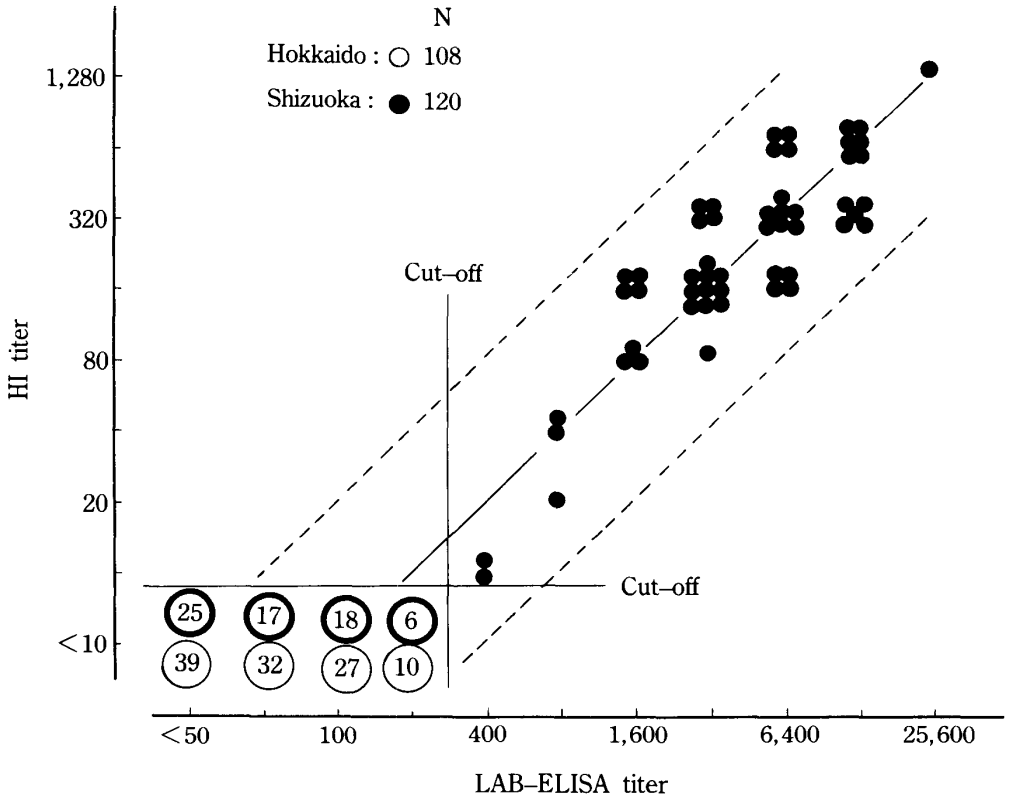
FIGURE 3 *Comparison of JE antibody titers by LAB-ELISA with ABTS and HI test in the sera from an experrimentally infected swine, No.* 1



LAB-ELISA with ABTS was applied to detect JE antibodies in the sera collected progressively from swine No. 1 (fig. 3). IgM or Ig antibody titers were compared with HI titers before and after 2-ME treatment. IgM antibody was detected first on day 6 after inoculation, reached peak titer at 1 : 1,280 on day 9 and decreased rapidly but maintained the titer at 1 : 40, even up to day 79. The Ig antibody was also detected on day 6 and reached a peak titer at 1 : 10,240 on day 28, and maintained the titer at 1 : 5,120 until day 79. HI antibody appeared first on day 6 and reached a peak at 1 : 640 on day 12. HI antibody resistant to 2-ME was detected on day 7 and reached a peak titer at 1 : 640 on day 21. However, the pattern of appearance of LAB-ELISA Ig antibody was the same as that of the HI antibody, and the LAB-ELISA Ig titers were 16 times higer than HI titers. On the other hand, the IgM antibody titers corresponded well with 2-ME sensitive HI titers in the early period of infection, but decreased when 2-ME resistant HI titers increased in the convalescence period. These results indicated that the Ig conjugate reacted to both IgM and IgG antibodies but that the anti-IgM conjugate reacted specifically against IgM antibody.

JE antibody titers by LAB-ELISA with ABTS using anti-Ig conjugate were compared with those by HI test in swine sera from Shizuoka prefecture and northern Hokkaido (fig. 4). All 108 sera from northern Hokkaido were negative (<1 : 10) in HI test and had LAB-ELISA Ig titers lower than 1 : 400. From Shizuoka prefecture, all 66

FIGURE 4 *Comparison of JE antibody titers in LAB–ELISA with ABTS by using anti–Ig conjugate and HI test in swine sera from Shizuoka and northern Hokkaido*

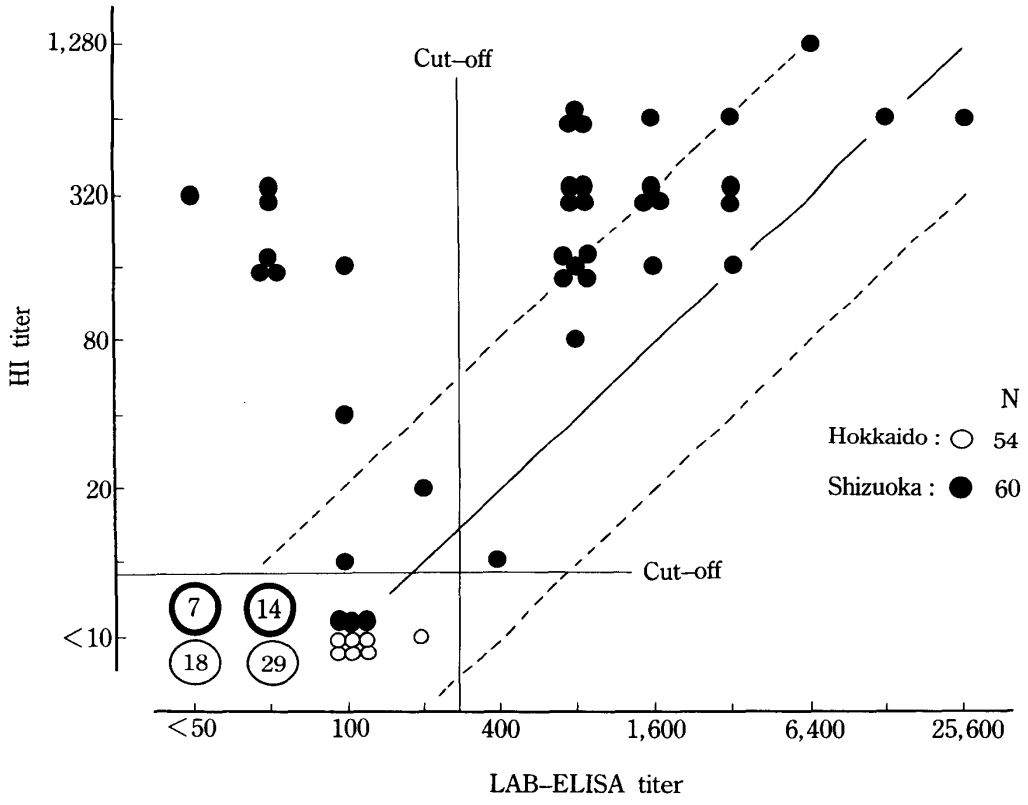


Numbers in these black circles indicate numbers of swine sera from Shizuoka and these in white circles for Hokkaido.

HI-negative sera had LAB-ELISA Ig titers lower than 1 : 400, and all 54 HI-positive sera had LAB-ELISA Ig titers 1 : 400 or more. Thus, the cut-off titer was set at 1 : 400 of serum dilution for positive Ig antibody in LAB-ELISA. Close correlation existed in HI and LAB-ELISA titers : the correlation coefficient was $r = 0.988$ The LAB-ELISA Ig titers were almost 20 times higher than the HI ones.

LAB-ELISA IgM titers to JE with ABTS were compared with HI titers of swine sera from Shizuoka prefecture and northern Hokkaido (fig. 5). All 54 sera of Hokkaido were negative in HI test and had a LAB-ELISA IgM titer lower than 1 : 400. Of the sera from Shizuoka prefecture, all 24 HI-negative sera had LAB-ELISA IgM titers lower than 1 : 400. Thus, the cut-off titer was also set at 1 : 400 for positive level of IgM antibodies in LAB-ELISA. Of 36 HI-positive sera ($\geq$ 1 : 10), 10 sera (27.8%) were negative in LAB-ELISA using anti-IgM conjugate. It was suspected that these sera contained IgG antibody, since HI test could detect it. Comparison of the HI titers with the LAB-ELISA IgM titers showed that there was no correlation between the 2 titers.

FIGURE 5 *Comparison of JE antibody titers in LAB-ELISA with ABTS by using anti-IgM conjugate and HI test in swine sera from Shizuoka and northern Hokkaido*

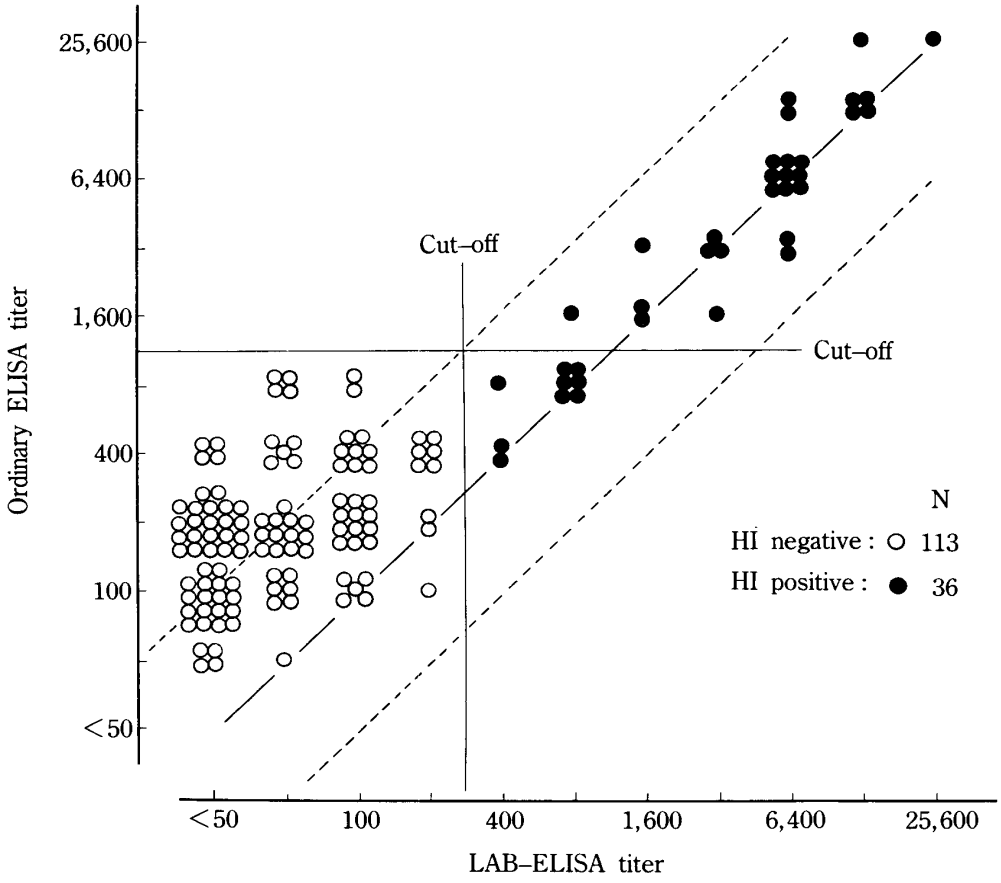


Numbers in these black circles indicate numbers of swine sera from Shizuoka and these in white circles for Hokkaido.

JE Ig antibody titers were compared in ordinary ELISA and LAB-ELISA by using ABTS (fig. 6). Most of the HI-positive sera had equal Ig antibody titers in both LAB-ELISA and ordinary ELISA. All 36 HI-positive sera had LAB-ELISA Ig titers 1 : 400 or more and all 113 HI-negative sera had LAB-ELISA Ig titers lower than 1 : 400. These results corresponded well with the decided cut-off titer (1 : 400) in the LAB-ELISA, as shown in Fig. 4.

On the other hand, ordinary ELISA titers in all 113 HI-negative sera distributed up to 1 : 800, but 27 out of 36 (75%) HI-positive sera had a titer higher than 1 : 1,600. Therefore, the cut-off titer was set at 1 : 1,600 for positive level of Ig antibody in ordinary ELISA. However, 9 out of 36 (25%) HI-positive sera were judged as negative by ordinary ELISA. Most of the HI-negative sera had ordinary ELISA Ig titers which were approximately 4 times higher than those of LAB-ELISA. Since LAB-ELISA had a less non-specific reaction, the assay could lower the cut-off titer more than that of ordinary ELISA. Thus, LAB-ELISA could increase the true

FIGURE 6 *Comparson of JE antibody (Ig) titers by LAB–ELISA and ordinary ELISA with ABTS in the swine sera*



Each symbol shows an individual serum of which HI titer is negative ○, and positive ●.

antibody rate, although the sensitivity of both assays for antibody detection was essentially the same.

DISCUSSION

In this study, it was demonstrated that LAB-ELISA with ABTS as a new substrate had a high sensitivity and a less non-specific reaction to detect Ig or IgM JE antibodies in swine sera.

The anti-IgM (μ chain specific) conjugate could detect only IgM antibody of JE. The antibody titers in LAB-ELISA with ABTS were approximately 20 times higher than the HI titers in sera from both an experimentally infected and a naturally infected swine. Although the sensitivities were almost equal in both LAB and ordinary ELISA using ABTS, LAB-ELISA had several advantages over ordinary ELISA. Since a less non-specific reaction was observed in LAB-ELISA, the cut-off titer for positive antibody could be set at a lower titer (1 : 400) than that (1 : 1,600) of ordinary ELISA.

Thus, LAB-ELISA could detect the antibody with a low titer which might be misjudged as negative in ordinary ELISA.

Biotin could be purchased as BHSE, which can bind actively to protein. BHSE binds stably to amino residue of Ig molecule, which facilitates prolonged preservation. Avidin binds specifically to biotin molecule, and the binding capacity between them is known to be higher than that between antibody and antigen.[5] Labeling of immunoglobulin with biotin was simple and reproducible, as compared with the conjugation procedure of immunoglobulin with HRP. Moreover, the labeling procedure did not require column chromatography.

Since multi-steps of reaction were involved in LAB-ELISA, standardization of each reagent was critical for correct estimation of antibody titers. Successful standardization of each reagent in LAB-ELISA allowed highly reproducible results. End point titers of reagents in both LAB- and ordinary ELISA with ABTS were about 4 times higher than those obtained with 5-AS. The coloring reaction with ABTS could be easily read by the naked eye. Thus, ABTS was beneficial for saving the reagents, and further, it was suitable for use in a mass serological survey of JE infection.

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