Studies on the Development of an ELISA Kit for Microbiological Monitoring

1. Evaluation of the Reliability of the Prototype Kit by Field Tests

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(Received 5 July 1989/Accepted 22 September 1989)

The prototype of an ELISA kit using protein A as the second reaction reagent for mice and anti-rat IgG for rats was prepared for seromonitoring of the Sendai virus and mouse hepatitis virus (MHV)/sialodacryoadenitis virus (SDAV)/Parker's rat coronavirus (PCV) infections. The respective antigen strains and protein concentrations were Sendai virus MN strain, 2 µg/ml and MHV Nu-67 strain, 5 µg/ml. The reliability of this prototype kit was investigated in two field tests performed on a total of 10,094 mouse and rat sera from 147 institutions. The results indicated that the two types of kits for the two species of animals were highly specific, but it is necessary to increase the detection sensitivity of the MHV antigen for the MHV antibody of mice and SDAV/PCV antibodies of rats.

The goal of this research is to develop an antibody test kit with excellent practicability in comparison with the methods widely used to date in the field of laboratory animal science, and make it available for in-house monitoring.

ELISA using enzyme-conjugated protein A as the second reaction reagent was selected as the technique best suited to the above purpose. When protein A is used in the second reaction, it is expected not necessary to exchange the reagent for such species as mice, hamsters, guinea pigs and rabbits [6]. Since protein A shows weak binding with the IgG of rats, anti-rat IgG was used instead of protein A for rats.

To assure that ELISA can be performed anytime, anywhere by anyone, it is essential to develop a kit which contains all of the antigens, antisera and reagents and which permits macroscopic evaluations.

In the present study, the development of a ELISA kit for seromonitoring of Sendai virus

(SV) and mouse hepatitis virus (MHV)/sialodacryoadenitis virus (SDAV)/Parker's rat coronavirus (PCV), the most prevalent in Japan [4] and highly pathogenic for mice and/or rats, was investigated. The prototype kit prepared on the basis of research results in our laboratory was subjected to field tests with the cooperation of nation-wide institutions, and the reliability of the kit were evaluated.

Materials and Methods

Antigen strains : As in the previous report, the MN strain was used for SV [8]. For detection of MHV antibody in mice and SDAV/PCV antibodies in rats, the MHV Nu-67 strain which has been reported to be useful for microbiological monitoring on these viral infections [7] was used.

Antigen preparation methods : SV antigen

solubilized in carbonate-bicarbonate buffer (pH 9.8) (CBB) was prepared by the procedures reported previously [8]. Protein concentrations of $2\mu\text{g/ml}$ were used for the antigen. For MHV, the virus strain was propagated in DBT cells and the microcarrier method [3] was employed for the massculture. Cytodex® 2 (Pharmacia) was placed as microcarrier into a 500ml spinner type culture bottle (Culstir; Shibata Hario Glass Co. Ltd.), and the cell culture was performed at 37°C using Auto-Pow® MEM medium (Flow Laboratories, Inc.) with 10% fetal calf serum (FCS) added in a 5% CO_2 atmosphere. MHV was inoculated on the 2nd day in FCS free medium. The culture supernatant was centrifuged at 3,000 rpm for 10 minutes and the supernatant obtained was again centrifuged at 20,000 rpm for 60 minutes. The precipitate was resuspended in PBS⁻, and the antigen was solubilized by adding 5-fold the amount of CBB. The protein concentration of the antigen was adjusted to $5\mu\text{g/ml}$.

ELISA: The procedures of ELISA were the same as previously reported [8].

Preparation of the kit: Kits of antibody testing of SV and MHV/SDAV/PCV were prepared. Each of them contained in addition to the plate for the solid phase of the antigen and the positive control sera for mice and rats, horse radish peroxidase (HRPO) (Toyobo, grade I-C)-conjugated protein A (Sigma Chemical, P 6650), HRPO-conjugated anti-rat IgG, *o*-phenylenediamine-2 HCl (OPD) (Nakarai Tesque, Inc.), hydrogen peroxide solution (Mitsubishi Gas Chemical), 4 N- H_2SO_4 to terminate the reaction, washing buffer ($5\times\text{PBS}^-$), sample dilution buffer ($5\times 0.5\%$ BSA-PBS⁻), solvent for enzyme-conjugated protein A and anti-rat IgG (1% BSA-PBS⁻) and purified water for the OPD solvent. The positive control serum was adjusted to show the absorbance value of 0.3-0.4 at 492nm.

Field tests on the prototype kits: The field tests were performed two times with the cooperation of a total of 147 institutions including 51 universities, 30 research institutes, 50 pharmaceutical companies and 16 breeders. The first test was undertaken in 1985 with the cooperation of 67 institutions on mice. The solid phase of SV or MHV antigen was prepared each in 96-well plate (Costar Corporation, U. S. A.). The second test was performed in 1986 with the cooperation of 80 institutions on mice and

rats. The plates were divided into two parts and the solid phases of the SV and MHV antigens were prepared in 48 wells each. The prototype kits were kept cool and sent to the cooperating institutions. In the cooperating institutions, the sera collected from their mice and rats were tested. Evaluation was made macroscopically. Chromogenesis equivalent to or stronger than that of the positive control was regarded as positive.

We received the test sera from the cooperating institutions, performed ELISA using prototype kits of the same lot and obtained the absorbance values by spectrophotometry (MTP-12 2-wavelength microplate photometer; Corona Electric). The reliability assessment of the macroscopic evaluations performed in comparison with the instrument evaluation as standard with absorbance value of 0.3 or higher at 492 nm regarded as positive. Analysis of histograms on absorbance value distribution against MHV antigen was also performed for serum samples collected during the second field test.

Results

First field test: SV and MHV antibodies were tested using a total of 6,745 samples of mouse serum. The serum dilution rate was 1:40. Among 3,337 samples, 2,952 were negative and 385 were positive for SV in the instrument evaluation (Table 1). Among the 2,952 negative samples, 2,879 (97.5%) were negative macroscopically, and 369 of the 385 positive samples (95.8%) were evaluated as positive macroscopically. The results of the macroscopic evaluation were in good agreement with those of the instrument evaluation. The reason for these results is that, since 2,861 (96.9%) of the negative samples showed no chromogenicity at 0.1 and 305 (79.2%) of the positive samples showed 0.5 higher absorbance values, macroscopic differentiation between negative and positive results was easy.

The same investigation was performed for MHV as for SV (Table 2). Among the 2,731 samples evaluated as negative by instrument evaluations, 2,463 (90.2%) showed matching macroscopic evaluations, but the matching rate for positive samples was only 71.6% (485/677). These results indicated that macroscopic evaluation was not as easy as in the case of

Table 1. ELISA on Sendai virus antibody in mice (Field test I)

Macroscopic evaluation	Instrument evaluation (absorbance value at 492nm)**					
	-			+		
	ABS* < 0.1	0.1 ≤ ABS < 0.2	0.2 ≤ ABS < 0.3	0.3 ≤ ABS < 0.4	0.4 ≤ ABS < 0.5	0.5 ≤ ABS
-	2,894	2,879(97.5%)			15(3.9%)	
	2,846	28	5	4	5	6
±	15	14(0.5%)			1(0.3%)	
	8	3	3	0	0	1
+	428	59(2.0%)			369(95.8%)	
	7	12	40	39	32	298
Total	3,337	2,952(100%)			385(100%)	
	2,861	43	48	43	37	305

* Absorbance value ** Positive control sera showed absorbance values of 0.333 ± 0.024 (N=12).

Table 2. ELISA on mouse hepatitis virus antibody in mice (Field test I)

Macroscopic evaluation	Instrument evaluation (absorbance value at 492nm)**					
	-			+		
	ABS* < 0.1	0.1 ≤ ABS < 0.2	0.2 ≤ ABS < 0.3	0.3 ≤ ABS < 0.4	0.4 ≤ ABS < 0.5	0.5 ≤ ABS
-	2,573	2,463(90.2%)			110(16.3%)	
	2,184	200	79	48	27	35
±	233	151(5.5%)			82(12.1%)	
	48	61	42	28	16	38
+	602	117(4.3%)			485(71.6%)	
	28	30	59	62	63	360
Total	3,408	2,731(100%)			677(100%)	
	2,260	291	180	138	106	433

* Absorbance value ** Positive control sera showed absorbance values of 0.377 ± 0.025 (N=12).

SV, since 318 samples (9.3%) showed weak chromogenicity of the cut-off absorbance range of 0.2 to 0.4.

Second field test : Antibody tests with SV and MHV antigens were performed on 2,028 samples of mouse sera and 1,321 samples of rat sera. The serum dilution was changed to 1 : 20. In mice, the matching rate for the results of the instrument and macroscopic evaluations of the SV antigen was 88.8% (882/993) for nega-

tive samples and 76.8% (133/173) for positive samples (Table 3). The results were not as good as those of the first field test in both cases. Unlike in the first test, the percentage of samples showing absorbance value lower than 0.1 among the negative samples was a rather low value of 88.9% (883/993). The reason for this phenomenon seemed to be that weak nonspecific chromogenicity appeared in the negative samples because of higher serum

Table 3. ELISA on Sendai virus antibody in mice (Field test II)

Macroscopic evaluation	Instrument evaluation (absorbance value at 492nm)**					
	-			+		
	ABS* < 0.1	0.1 ≤ ABS < 0.2	0.2 ≤ ABS < 0.3	0.3 ≤ ABS < 0.4	0.4 ≤ ABS < 0.5	0.5 ≤ ABS
-	914	882(88.8%)			32(18.5%)	
		768	99	15	9	14
±	65	57(5.8%)			8(4.6%)	
		33	18	6	4	1
+	187	54(5.4%)			133(76.9%)	
		32	16	6	5	122
Total	1,166	993(100%)			173(100%)	
		833	133	27	18	137

* Absorbance value ** Positive control sera showed absorbance values of 0.272 ± 0.025 (N=12).

Table 4. ELISA on mouse hepatitis virus antibody in mice (Field test II)

Macroscopic evaluation	Instrument evaluation (absorbance value at 492nm)**					
	-			+		
	ABS* < 0.1	0.1 ≤ ABS < 0.2	0.2 ≤ ABS < 0.3	0.3 ≤ ABS < 0.4	0.4 ≤ ABS < 0.5	0.5 ≤ ABS
-	508	489(69.2%)			19(12.3%)	
		407	59	23	5	11
±	111	90(12.7%)			21(13.5%)	
		62	14	14	16	2
+	243	128(18.1%)			115(74.2%)	
		44	39	45	31	62
Total	862	707(100%)			155(100%)	
		513	112	82	52	75

* Absorbance value ** Positive control sera showed absorbance values of 0.272 ± 0.025 (N=12).
The values shown for the Sendai virus positive control sera were applied in the evaluation of both Sendai virus and mouse hepatitis virus antibodies.

dilution rate of 1 : 20. As a result, the cut-off range became unclear and among the positive samples, it appeared that the percentage evaluated as negative macroscopically increased to 18.5% (32/173).

In the case of MHV antigen, the matching rate of macroscopic evaluations for 707 samples evaluated as negative by instruments showed a marked decrease to 489 (69.2%) (Table 4). The

reason for this was that the samples evaluated macroscopically as positive accounted for 18.1% (128/707) of all negative samples even the absorbance values showed 0.3 or lower. It was assumed that the blurring of the cut-off range by weak nonspecific chromogenicity described above was involved.

In the case of rats, 544 (90.7%) of the 600 which were SV antibody negative according to

Table 5. ELISA on Sendai virus antibody in rats (Field test II)

Macroscopic Evaluation	Instrument evaluation (absorbance value at 492nm)**					
	-			+		
	ABS* < 0.1	0.1 ≤ ABS < 0.2	0.2 ≤ ABS < 0.3	0.3 ≤ ABS < 0.4	0.4 ≤ ABS < 0.5	0.5 ≤ ABS
-	571	544(90.7%)			27(32.1%)	
		399	123	22	8	11
±	16	10(1.7%)			6(7.2%)	
		1	2	7	1	4
+	97	46(7.6%)			51(60.7%)	
		17	13	16	7	9
Total	684	600(100%)			84(100%)	
		417	138	45	16	21

* Absorbance value ** Positive control sera showed absorbance values of 0.298 ± 0.022 (N=12).

Table 6. ELISA on sialodacryoadenitis virus/Parker's rat coronavirus antibodies in rats (Field test II)

Macroscopic evaluation	Instrument evaluation (absorbance value at 492nm)**					
	-			+		
	ABS* < 0.1	0.1 ≤ ABS < 0.2	0.2 ≤ ABS < 0.3	0.3 ≤ ABS < 0.4	0.4 ≤ ABS < 0.5	0.5 ≤ ABS
-	548	524(92.1%)			24(35.3%)	
		436	61	27	2	3
±	35	16(2.8%)			19(27.9%)	
		8	2	6	3	2
+	54	29(5.1%)			25(36.8%)	
		6	8	15	3	0
Total	637	569(100%)			68(100%)	
		450	71	48	8	5

* Absorbance value ** Positive control sera showed absorbance values of 0.298 ± 0.022 (N=12).

The values shown for the Sendai virus positive control sera were also applied in the evaluation of sialodacryoadenitis virus/Parker's rat coronavirus antibodies.

the instrument evaluation were negative macroscopically ; the matching was good (Table 5). However, the matching rate for 84 positive samples was low, only 60.7% (51/84). The problem was that the samples evaluated macroscopically as negative at absorbance value of 0.3 or higher accounted for 27(32.1%) of the 84 positive samples. The reason for this

appeared to be that weakly positive samples among those with strong chromogenicity at absorbance value of 1.0 or higher might be read as negative for a visual illusion. This tendency was more marked in the SDAV/PCV antibody tests (Table 6), i. e., 24 (35.3%) of the 68 samples found to be positive in the instrument evaluation were evaluated as negative macrosc-

Fig. 1. Distribution of absorbance values in ELISA on mouse hepatitis virus antibody in mice (Field test II)

Fig. 2. Distribution of absorbance values in ELISA on sialodacryoadenitis virus/Parker's rat coronavirus antibodies in rats (Field test II)

opically, and 19 of these samples showed chromogenicity at 0.5 or higher.

Analysis of problems based on absorbance value distribution: Histograms of absorbance values for MHV antigen of the prototype kit prepared for the second field test were obtained (Figures 1 and 2), and the reason for the poor matching between the instrument and macroscopic evaluations was analyzed. They include

absorbance values of the additional 382 mouse and 318 rat sera (1,244 and 955 in totals, respectively) obtained from some institutions included in Tables 4 and 6 for confirmation of the results obtained in this study. The results indicated that 145 (11.7%) of the 1,244 samples from mice were distributed in the cut-off absorbance range of 0.2-0.4. There was a peak of absorbance value in negative region at 0.0-0.2, but no such peak was seen in the positive region, and the distribution near the cut-off range showed a gentle slope.

Fig. 2 shows a histogram concerning the reaction of rat sera. Among a total of 955 samples, 69 (7.2%) were in the absorbance 0.2-0.4 range and the trends in frequency distribution were the same as those for mice.

Discussion

Prototype antibody test kits were prepared for four pathogens which present major problems in mouse and rat colonies in Japan: SV, MHV and SDAV/PCV. The kit were prepared on the basis of results obtained in our laboratory and were devised to permit macroscopic evaluations to increase their practicability. Field tests were performed to determine whether or not reliable macroscopic evaluation results can be obtained by persons who are not experts in microbiology.

In the two field tests, six antibody test results were obtained from various combinations of animal species and antigens. For SV, there were no significant differences between the instrument and macroscopic evaluations, and basically good evaluations could be made with respect to both sensitivity and specificity.

The major problem with the prototype kit concerned the MHV antigen, i. e., there was no clear peak in the absorbance value distribution in the positive region, and a high percentage of samples (7-12%) showed absorbance values in the cut-off range of 0.2-0.4. However, since there was a clear peak in the negative region, there seemed no problem concerning the specificity of the reaction, and it was assumed that the cause was the sensitivity.

It was demonstrated that there was a RNA and polypeptide homology among murine coronaviruses [1]. We used the Nu-67 strain as the MHV antigen because MHV infection could be detected efficiently for six prevalent mouse

strains [5] and there was also a good reaction with SDAV/PCV infected rat sera with respect to complement fixation test [7]. However, it will be necessary to reconsider the strain used as MHV antigen, since different antigen determinants have been demonstrated among the virus strains [2]. The antigen titer should also be reexamined at the same time. Low serum dilution of 1 : 20 in the second field test might have affected the blurring of the cut-off range with weak chromogenicity.

In these two test, there were examples of weak chromogenicity at absorbance value of 0.2-0.4 for some of the sera of aged rats from breeder colonies considered to be free from the three pathogens involved in this study (data not shown). Therefore, the aging factors of the animals will also have to be studied in the future.

In mouse models for autoimmune diseases such as MRL/l pr and NZB/NZWF 1, there have been occasional cases of nonspecific chromogenicity at absorbance value of 1.0 or higher in this study associated with increases in immunoglobulin concentration in the sera. Therefore, it is necessary to prepare sentinel mice when the ELISA kit is used in monitoring of these special strains of mice. Future topics for improvement of the kit include reconstruction of the plates so that the color of adjacent wells will not be reflected as a means to prevent visual errors at the time of macroscopic evaluations.

This study was partially supported by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan (No. 60880012).

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微生物モニタリング用 ELISA キットの開発

1 野外試験による試作キットの信頼性評価

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センダイウイルス (SV) とマウス肝炎ウイルス (MHV)/唾液腺腺炎ウイルス (SDAV)/Parker ラットコロナウイルス (PCV) の抗体検査用 ELISA キットを試作した。2 次反応試薬として、マウス検査用にはプロテイン A を、ラットには抗ラット IgG を使用し、SV MN 株と MHV Nu-67 株をそれぞれ蛋白濃度 $2\mu\text{g/ml}$ と $5\mu\text{g/ml}$ に調整して固相化抗原とした。判定は肉眼で実施することとした。試作キットの信頼性評価のため、

147機関の協力を得て、10,094検体のマウス、ラット血清を材料とする野外試験を実施した。その結果、2種のキットはいずれも特異性にすぐれていたが、MHV を抗原とするキットについては、マウスの MHV 抗体、ラットの SDAV/PCV 抗体のいずれに対しても検出感度が不十分であり、抗原の改良が必要であると考えられた。