An Application of Immuno Peroxidase Staining Technique for the Titration of Dengue Virus *in vitro*

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SUMMARY

A newly modified enzyme focus forming assay (EFFA) has been developed for the titration of dengue viruses (DV). Infectious foci were stained with peroxidase labeled antibody and colorized with Karnovsky's solution. The foci were easy to count by the naked eye and were semipermanently preserved. The EFFT was compared either with conventional plaque assay or with suckling mouse intracerebral inoculation for the sensitivity and for the incubation period. The EFFT was higher in sensitivity and reduced the incubation period more than 2 days in DV titration. Therefore the EFFT was useful for the titration especially of non/weak-cytopathogenic viruses. The method was also useful for the ultrastructural study of viral replication in electron microscopy. The variables affecting the assay were investigated.

INTRODUCTION

The plaque formation in viral infectious experiments has been utilized not only for the viral titration but also for the detection of mutant strains such as pathogenic variants. However, when the cytotoxicity is weak or absent in the virus-host combination, it is difficult to form plaques. And the plaque formation requires longer incubation for titration of such viruses as dengue virus (DV)¹⁴⁾.

To overcome this difficulty, immunocytochemical techniques have been applied. Igarashi and Mantani⁷⁾ have developed a new method of counting fluorescent foci on the virus infected cultured cells. Although their method is useful in titrating the viral infectivity and save the incubation time, it has some disadvantages. A fluorescence microscope is necessary and it is useful only for the analysis of the viruses which spread by the cell to cell infection.

We have now developed a new method, the Enzyme focus forming assay (EFFA), in which we combined the plaque forming assay (PFA)^{2) 5) 17)} and the enzyme labeled antibody assay system^{13) 14)}. The EFFA enable one to resolve the above mentioned problems and has more advantages for the morphological study^{12) 18)}.

In the present study, we have compared the results of our new method with those of the previous conventional methods using Japanese encephalitis virus (cytopathogenic virus ; JEV), and DVs (weak cytopathogenic viruses). The variables affecting the analysis have been investigated and some experiments are presented.

MATERIALS AND METHODS

Cells

Vero cells were subcultured in Eagle's minimum essential medium (MEM), supplemented with 10% calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). For the virus inoculated cells, 2% calf serum was supplemented to MEM.

Viruses

JEV, JaGAr-01 strain, was obtained from Dr. T. Kurimura*, Osaka Prefectural Institute of Public Health. DVs, type I (D-I) Hawaii strain, type II (D-II) Tr 1751 strain, type III (D-III) H-87 strain and type IV (D-IV) H-241 strain, were obtained from Dr. K. Fukai, Institute of Microbial Diseases, Osaka University. All viruses in suckling mouse brain (SMB) were passed either in SMB or in Vero cells for 5 times. Ten percent of infected SMB emulsion was prepared with Hanks' balanced salt solution supplemented with 0.5% bovine serum albumin (Fraction V, Sigma, St. Louis, MO), penicillin and streptomycin at 100 U/ml and 100 µg/ml, respectively (HBSA). The emulsion was centrifuged at 5,000 rpm for 5 min at 4°C. The supernatant was distributed and stored at -80°C until use. Virus infected Vero cells were frozen and thawed for 3 times using dryice-acetone (-70°C) and water, then they were centrifuged at 5,000 rpm for 5 min at 4°C. Each supernatant was distributed and stored at -80°C until use.

Antisera

Antiserum to each virus was prepared in male white rabbits. The procedures have been described elsewhere⁸⁾. The immunoglobulin G (IgG) fraction was precipitated with the 1/3 saturated ammonium sulfate. IgG was absorbed nonspecific agents with Vero cell acetone powder and mouse liver acetone powder. Horseradish peroxidase (HRPO) labeled anti-rabbit IgG goat IgG (Miles-Yeda, Israel) was used for the second serum in the indirect method.

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Virus infection

Vero cells were suspended in MEM (2×10^5 cells/ml) and were dispensed into wells of plastic multiwell plate (4×6 wells, Nunc, Denmark) for 1 ml each. The plates were incubated at 37°C in a CO_2 incubator. A 0.1 ml of serially ten fold diluted virus suspension was absorbed for 90 min at 37°C . After the incubation, 1 ml of the overlay medium, containing 1.0–1.8% methylcellulose and 2% calf serum, was added to each well. Plates were incubated in a CO_2 incubator for 2 to 7 days.

Plaque assay

After the suitable incubation, the overlay medium was removed. The cells were washed with cold PBS and were fixed with cold methanol for 10 min. The wells were divided into two groups, one for the PFA and the other for the EFFA. In the PFA, the cells were stained with Giemsa solution.

Focus staining method with enzyme labeled antibody

The flow chart of the EFFA was shown in Table 1. A drop of goat normal serum (1 : 50) was mounted on the fixed cells of each well to prevent nonspecific adsorption of HRPO labeled IgG. Then a 0.1 ml of anti-viral IgG suspension, diluted with PBS for appropriate concentration, was added to each well. HRPO labeled anti-rabbit IgG goat IgG was used in the second reaction. In every step, plates were incubated at 37°C for 30 min in a moist chamber. After the incubation, wells were washed with PBS which was supplemented with

Table 1. Flow chart describing the focus-staining method using peroxidase labeled antibody (Enzyme Focus Forming Assay ; EFFA)

Multiwell plate with the confluent Vero cell monolayer.
 Infect with serially diluted virus.
 Incubate at 37°C for 3 to 7 days with 5% CO_2 .
 Remove overlay medium and wash each well 3 times with cold PBS at 4°C and blot.
 Fix with cold methanol (-20°C) for 10 min.
 Dispense normal goat sera to each well^a and incubate at 37°C for 30 min.
 Wash 3 times for 15 min each time with cold PBS/T/BSA^b at 4°C .
 Dispense the dilute primary sera^c to each well and incubate at 37°C for 30 min.
 Wash 3 times for 15 min each time with cold PBS/T/BSA at 4°C .
 Dispense the dilute secondary sera^d to each well and incubate at 37°C for 30 min.
 Wash 3 times for 15 min each time with cold PBS/T/BSA.
 Stain with Karnovsky's solution^e at room temperature.

a : 0.1 ml/well for 24 wells ($\phi = 16$ mm) plate.

b : PBS, pH 7.2 with 0.05% Tween-20 and 0.5% bovine serum albumin.

c : Anti-virus rabbit IgG.

d : HRPO labeled anti-rabbit IgG goat IgG. (Miles-Yeda, Israel).

e : 0.05M Tris-HCl buffer, pH 7.6 with 0.02% 3,3'-diaminobenzidine 4 HCl and 0.01% H_2O_2 .

0.05% Tween-20 and 0.5% bovine serum albumin (PBS/T/BSA) for 3 times for 15 min each time. For colorizing the infectious foci Karnovsky's substrate solution⁴⁾ was used and kept at room temperature (Table 1).

Mouse intracerebral inoculation

Serially 10 fold diluted DVs were inoculated to 1-2 days old suckling mice intracerebrally⁶⁾. Fifty percent lethal dose (LD₅₀) was calculated by Reed and Muench¹⁶⁾.

Electron microscopy of the infectious foci

Specimen for the electron microscopy was prepared with a slight modification in fixation. After incubation for 4 days, the foci of D-II infection were prefixed at 4°C for 2 h with periodate-lysine-paraformaldehyde¹¹⁾ which was supplemented with 0.1% glutaraldehyde¹⁸⁾. The immunoenzymatic process was followed by the method described above. But antisera were reacted at 4°C for overnight instead of the incubation at 37°C for 30 min. Foci were post fixed with 1% OsO₄ at 4°C for 2 h, were dehydrated and were embedded in Epon 812 resin¹⁰⁾. The virus infected cells were easily trimmed under a converted microscope. Ultrathin sections were observed with or without staining with uranyl acetate and lead citrate³⁾. They were observed under a Hitachi electron microscope model H 500 operating at 100kv.

RESULTS

Comparative sensitivity of the EFFA for titration of JEV

Plaque or focus size, incubation period and viral titers as detected by the PFA or by the EFFA were compared on JEV. Concentration of methylcellulose (MC) was 1.5% both in the EFFA and in the PFA according to Hsue⁵⁾. By the PFA, plaques were detected after incubation for 4 days with the size of 0.2-0.4 mm in diameter. The size enlarged to 0.6-0.8 mm in 5 days of incubation. While the infectious focus in the EFFA was observed 2 days after inoculation and the diameter was about 0.2 mm. After incubation for 5 days, the focus was a doughnut-like shape, with 0.15-0.2 mm in width and 1.2-1.4 mm in the outer diameter (Fig. 1). Microscopic observation of this focus showed that the central less dense area was based on the virus cytopathic effect (CPE) and that the cells were stripped off from the plate. While the marginal zone of the focus was partially healthy in spite of the virus infection. Therefore the cells were stained as antigen positive (data is not shown).

The prolonged incubation affected only the focus size and did not the focus number. The viral titer was kept in the same level through the incubation time. There were no differences in the infectious titer of the original JEV in SMB between two assay system (Table 2 and Fig. 1).

Table 2. The comparison of viral titer and focus size between the EFFA and the PFA on Vero cells after JEV inoculation, and the effect of incubation time on them

		Days after inoculation					
		1	2	3	4	5	7
Plaque assay	Plaque size (mm)	NT ^{a)}	invisible	invisible	0.2—0.4	0.6—0.8	1.0—1.2
	viral titer (PFU/ml)	NT	—	—	2.0×10^7	1.8×10^7	2.1×10^7
EFFA assay	Focus size (mm)	NT	0.2	0.4—0.6	0.6—0.8	1.2—1.4	1.4—1.8
	Viral titer (EFFU/ml)	NT	1.8×10^7	2.2×10^7	2.0×10^7	2.0×10^7	1.9×10^7

a : Not tested.

Concentration of MC in the overlay medium

Effects of the concentration of MC on the EFFA was investigated (Table 3). After incubation for 3 days, the foci with diameters of 0.2 mm or less were easily observed in all strains of DV. Variations of the concentration of MC did not affect the focus size nor shape. But after incubation for 5 days, those incubated with 1.0% of MC showed unclear foci while those with 1.2—1.8% formed clearly defined foci with diameters of 0.6–0.8 mm. After incubation for 7 days, foci of 1.8% MC were slightly smaller than those of either 1.2% or 1.5% MC.

The incubation time and the focus size of DV

To obtain the optimum incubation time for the EFFA, the foci were fixed and stained at

Table 3. The effect of concentration of methylcellulose in the overlay medium on the focus size of DVs on Vero cell

Virus	Days after inoculation	Concentration of methylcellulose (%)			
		1.0	1.2	1.5	1.8
D-I	3	<0.2 ^{a)}	<0.2	<0.2	<0.2
	5	0.4—0.6	0.6—0.8*	0.6—0.8*	0.4—0.6
	7	1.0—1.6	1.4—1.6	1.2—1.4	1.2—1.4
D-II	3	0.2	0.2	0.2	0.2
	5	0.8—1.0	0.6—0.8*	0.6—0.8*	0.6—0.8
	7	1.2—1.6	1.2—1.4	1.6—1.8	1.0—1.2
D-III	3	<0.2	<0.2	<0.2	<0.2
	5	0.4—0.6	0.6—0.8*	0.6—0.8*	0.6—0.8
	7	1.4—1.6	1.2—1.4	1.2—1.4	1.0—1.2
D-IV	3	0.2	0.2	0.2	0.2
	5	0.6—0.8	0.6—0.8*	0.6—0.8*	0.6—0.8
	7	1.4—1.6	1.2—1.4	1.0—1.4	0.6—1.0

a : Focus size (mm).

* : Fine focus.

3, 5 and 7 days after DV inoculation (**Table 3**). After incubation for 3 days, foci of DVs were detected by the EFFA, though the diameter was smaller than 0.2 mm. The prolonged incubation caused larger foci in each strain of DV. After incubation for 5 days, foci were uniform and were easily countable (**Table 3** and **Fig. 2**). But foci were sometimes unclear after incubation for 7 days. On the other hands, no plaques were formed by the PFA until 7 days after inoculation in all DV strains used in this study (data are not shown).

Effect of host cell in which the test virus prepared

In applying the EFFA for viral titration, effect of host cell, in which the test virus multiplied, on the focus formation was investigated. As shown in **Table 4**, the difference of the viral source did not affect the focus formation by D-I and D-III or a little (D-II and D-IV).

Assay of infectious titer of DVs by the EFFA and comparison to suckling mouse brain inoculation assay

The EFFA was compared with suckling mouse brain inoculation assay (SMBA) for the sensitivity. Serially 10 fold diluted viruses which were passed in Vero cells were inoculated to Vero cells and into suckling mouse brain. Incubation time, required for the titration by both methods, were compared as shown in **Table 5**. In addition, the infectious titer by the EFFA (EFFU) and by the SMBA (LD_{50}) were compared on the aliquot of the material. The focus was detected after incubation for 3 days though the diameter was smaller than 0.2 mm. The infectious titer was about 10^8 EFFU/ml for 4 strains of DV. To the contrary, the mouse showed typical clinical signs 4 days after inoculation and died 5 to 6 days after inoculation. The LD_{50} was about 10^8 /ml for D-I, II and IV, 10^7 /ml for D-III.

Electron microscopy of the infectious focus

As shown in **Fig. 4**, characteristic tubular or small vesicular structures which sometimes appeared in the Togavirus infected cells were stained heavily on the cells located at the central zone of the foci. Some vesicles contained mature or budding viral particles.

On the surface of the cells which located marginal zone of the foci, viral particles attached to the cell membrane or penetrating particles through the coated pit were observed. And the cell surface was stained heavily.

Table 4. The comparison of the focus size between SMB passed and Vero cell passed DVs by the EFFA*

Virus	Source of viruses, replicated in	
	SMB	Vero
D-I	0.6—0.8 ^{a)}	0.6—0.8
D-II	0.4—0.6	0.6—0.8
D-III	0.6—0.8	0.6—0.8
D-IV	0.4—0.6	0.6—0.8

* : The infectious focus was stained 5 days after the incubation.

a : Focus size (mm).

Table 5. The comparison of sensitivity in viral titration between the EFFA and the SMB inoculation method*.

Days after inoculation	Focus size ^{a)}				Clinical sign ^{b)}			
	D-I	D-II	D-III	D-IV	D-I	D-II	D-III	D-IV
1	—	—	—	—	○	○	○	○
2	+	+	+	+	○	○	○	○
3	<0.2*	0.2	<0.2	0.2	○	○	○	○
4	NT	NT	NT	NT	S ₁₋₂	S ₁₋₂	S ₁₋₂	S ₁₋₂
5	0.6—0.8	0.6—0.8	0.6—0.8	0.6—0.8	S _{2-D}	S _{2-D}	S _{2-D}	S _{2-D}
6	NT	NT	NT	NT	D	D	D	D
7	1.4—1.6	1.2—1.4	1.2—1.4	1.2—1.4				
Titer ^{c)}	6.5×10^7	4.1×10^8	8.4×10^7	2.7×10^8	1.3×10^8	4.3×10^8	4.7×10^7	1.3×10^8

* : A 0.1 ml of serially ten fold diluted DVs was inoculated onto Vero cells and a 0.02 ml of the aliquot material was inoculated into suckling mice intracerebrally.

a : Focus size, diameter in mm ;

— : undetectable

+: detectable with a light microscope

* : pin point size

NT : Not tested.

c : EFFU/ml and LD₅₀/ml.

b : Clinical signs are as follows ;

○ : healthy

S₁ : suspected paralysis or piloerection

S₂ : paralysis of hind limbs

D : died.

DISCUSSION

Quantitative *in vitro* analysis in virology, such as the viral infectious titer and the neutralizing antibody titer, are usually expressed as the value of TCID₅₀, PFU and NT. But these methods are unsuitable for the assay of viruses which cause little or no CPE to the cultured cells. For example, plaque assay method takes longer incubation time^{14) 17)} in DV titration. Immunocytochemical method is another method to detect the viral infection. Since the first introduction of a fluorescent antibody method by Coons and Kaplan¹¹⁾, it has been developed remarkably. However, this technique has some disadvantages. The stained specimen loses the fluorescence in a short time, and it requires a fluorescence microscope to observe the specimen. Nakane and Pierce¹³⁾ developed the enzyme labeled antibody method. It is now as effective as the fluorescent antibody method owing to various improvements. In this method, specimens can be preserved semipermanently and requires only a conventional light microscope. Moreover, the location of the antigen can be examined with an electron microscope⁹⁾. However, these methods have not considered the viral titration.

Igarashi and Mantani⁷⁾ stained the focus produced by viral infection with fluorescent antibody, and the viral titer was measured by counting the stained foci under a fluorescence

microscope as the fluorescence focus forming unit (FFFU). Okuno *et al.*¹⁴⁾ modified the method to introduce an enzyme labeled antibody but their procedure had many steps and reagents. In their procedure, the foci must be counted in the small size. Therefore, it needs a light microscopic observation, and the observation is troublesome. Furthermore, it is useful only for the virus-cell system in which viral infection spread by cell to cell infection.

In the present study, we have stained the infectious focus with enzyme labeled antibody, by which the focus was counted easily with naked eye. This method is useful for any virus-cell system in which the virus replicate.

At first the EFFA was evaluated in its sensitivity, comparing with the PFA. For this evaluation, JEV was titrated because it was possible to assay the infectious titer by the both methods. When aliquots of the specimen were titrated by each method, the infectious titer was well coincided with each other. However, the focus size of the EFFA was larger than the plaque size of the PFA. Because, in the former system, marginal cells of plaques were in the early stage of the viral infection and were stained as viral antigen positive. Therefore foci were larger and were detected 1 to 2 days earlier than plaques.

Concentration of methylcellulose or agar in the overlay medium influences on plaque size and as the result, on the incubation time for plaque formation⁵⁾. In the present study, the concentration of MC in the overlay medium was better in range of 1.2—1.5% on the focus staining. From the reasons of sharpness, reproducibility of foci and handling easiness, 1.2% overlay medium was chosen in the following studies. This concentration was reasonable to the other report⁵⁾. By this concentration and incubation for 5 days, fine and reproducible foci of DV were obtained. But the plaque formation was insufficient during this incubation period. It took more than 7 days to form clear plaques under the MC concentration of 1.2—1.5%.

By the condition described above, infectious foci were detected after incubation for 3 days (Table 2 and 3). Focus size increased during the incubation. The focus was also detected on the 2nd day of incubation by light microscopy as reported by Igarashi and Mantani⁷⁾, and Okuno *et al.*¹⁴⁾. However, counting foci with the naked eye was more accurate and easier than the counting with a light microscope.

Focus size of DVs adapted in SMB was similar to that of DVs adapted to Vero cells (Table 4). D-II wild type strains, which were isolated from human patients and were passed in C6/36 cells as few as possible, were titrated by the EFFA. The precise data will be reported in the following paper. In brief, these wild type strains could be titrated by the EFFA but could not by the SMBA, because they could not cause lethal infection to the mouse. These results indicate that the EFFA does not select the sources of viral material,

therefore, is able to apply widely for the quantitation of wild type viruses.

The EFFA and the SMBA were compared for their sensitivity and the incubation time to estimate the viral titer. When an aliquot of DV specimen was inoculated into SMB, the mouse showed clinical signs 4 days after inoculation and died at 6 day. Thus, the infectious titer (LD_{50}) could be estimated later than the 6th day. While it took 3 days' incubation to calculate the infectious titer by the EFFA (EFFU). The infectious titers were almost equal in the both methods (**Table 5**).

Nakamichi¹²⁾ applied the EFFA for the morphological study of rubella virus (RV) replication. In that report he could count its infectious titer by the EFFA. This method was more simple and more rapid than the interference system of Parkman *et al.*¹⁵⁾ for RV titration¹²⁾.

Furthermore, the EFFA is applicable to the electron microscopy (IEM) changing the fixatives for the IEM^{12) 18)}. The specimen, prepared by this system has two advantages. 1) It is easy to approach to the virus infected cells, for they are positively stained by the immuno enzymatic staining and visible. 2) The viral infectious process is observed easily. The marginal area corresponds to the early stage of viral replication and the central area to the late stage of the replication.

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LEGEND FOR FIGURES

- Fig. 1 :** Titration of JEV by the EFFT (upper 2 rows) and by the PFA (lower 2 rows). A 0.1 ml of serially ten fold diluted virus was inoculated into each well. Immuno peroxidase positive infectious foci appear in a doughnut-like stain (upper 2 rows). Infectious foci are also countable by clear plaques (lower 2 rows). No differences of viral titer are found between the EFFT and the PFA. The plate was stained 5 days after inoculation. C : mock infection.
- Fig. 2 :** Titration of D-IV by the EFFT and by the PFA. Vero cell passed D-IV was serially tenfold diluted and a 0.1 ml of each was inoculated to each well. After 5 days of incubation, cells were stained either by EFFT (upper 2 rows) or by the PFA (lower 2 rows). Immuno peroxidase positive foci are stained homogeneously (not ring form, upper 2 rows), while plaques did not formed (lower 2 rows).
- Fig. 3 :** Micrographs of immuno peroxidase positive foci in the well. Three days (a) and 5 days (b) after D-II inoculation, the cells were stained. The foci were photographed from the backside of the plate. Scales indicate 0.5 mm.
- Fig. 4 :** Electron micrograph of a focus of D-II. Four days after inoculation, the infectious focus was fixed with 2% GA and immuno peroxidase stained by the EFFT. Tubular structures and vacuoles which are typically found in the togavirus infected cells, are heavily stained. Arrows indicate DV particles in the vacuoles. Scale indicates 500 nm.

Fig. 1

Fig. 2

Fig. 3

Fig. 4