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SHORT COMMUNICATION

A " SPOT-INFECTION TECHNIQUE " FOR TITRATION AND NEUTRALIZATION OF POLIOVIRUS[1]

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Two basic types of neutralization technique have been used. The first type is based on quantitative assay of virus, as in methods of plaque counting or end point tube dilution or in the microtitrator system (Takatsy, 1954; Gwaltney, 1966; Fuccillo, 1969). The second type is mainly based on the diffusibility of serum antibody and the permeability of virus into agar, as in serum diffusion methods (De Somer and Prinzie, 1957; Kalter, 1957; Wecker, 1960; Porterfield, 1960; Diwan et al., 1963; Umeda et al., 1963; Burt and Cooper, 1961; Wallis et al., 1965; Kiyota, 1968; Minamitani, 1968; Yoshino et al., 1971). The first type of neutralization method is more sensitive and gives accurate results, but requires much skill and many manipulations. The second type was developed as a simpler technique, but the most troublesome process of dilution has not been avoided. With the microtitrator system the procedure is improved by use of a diluter and microtransfer technique (Catalano, 1969). So this system has been widely used.

We have developed a simple micro neutralization system in poliovirus type 1 and Vero cells using a newly developed " spot-infection technique." This method does not involve dilution ($\leqq 1:256$) and can be done with samples of 0.02 ml of serum. In this system only a small area of monolayer in contact with the bottom of a hemispherical cup in an agar-covered-monolayer plate is infected with infectious virus.

Confluent monolayers of Vero cells were grown in Petri dishes (50 mm internal diameter, Toyoshima Co., Tokyo). Then 6 ml or less of molten medium consisting of equal volumes of Eagle's MEM and YLE containing 1.1% special agar-noble (Difco), 0.15% extra pure gelatin (Nakarai Chemicals) and 2.0% calf serum were poured into the dishes. One or 3 hemispheres (originally designed for this work as shown in Fig. 1 and named " Sitters ") were then placed in the dishes on the monolayers. The Sitters were gently removed after the agar had solidified. The cells in contact with the bottom of the hemispherical cups were thus left in agar cups in the plates. Microscopic examination showed that the cells were damaged, but they were completly repaired on subsequent incubation for 18 to 20 hr under 5% CO_2-air mixture at 37 C with 0.02 ml of growth medium (equal volumes of Eagle's MEM and YLE containing 10% calf serum) in the cups. The monolayers covered with agar,

1 This work was reported at the 20th Annual Meeting of the Society of Japanese Virologists, at Osaka, on November 4, 1972.

on which the cups were set to form the places for " spot-infection " were named " monolayer agar cup plates." Samples of 0.03 ml of virus (Mahoney strain) in PBS(—) containing 1.0% methylcellulose were placed in the agar cups using the droppers for the micro CF-test.

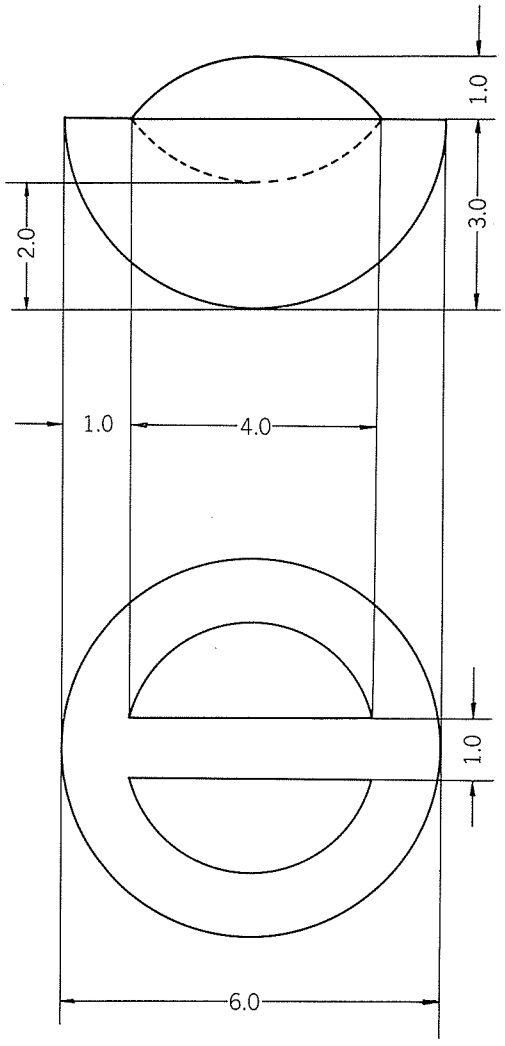

FIGURE 1. *Original design of a " Sitter ", from which metal moulds and Sitters made of silicon rubber were formed and used for preparing monolayer agar cup plates. Top, vertical section; Bottom, horizontal view of a Sitter. The knob, on top of the Sitter, is useful for removing the Sitter from the solid agar. The figure shows the dimensions of each part in mm.*

Good circular plaques were formed, and their area, expressed as the relative plaque area (product of two diameters measured at right angles to the nearest 0.05 mm using a slide caliper), increased in proportion to either the quantities of virus or the time after infection (Fig. 2).

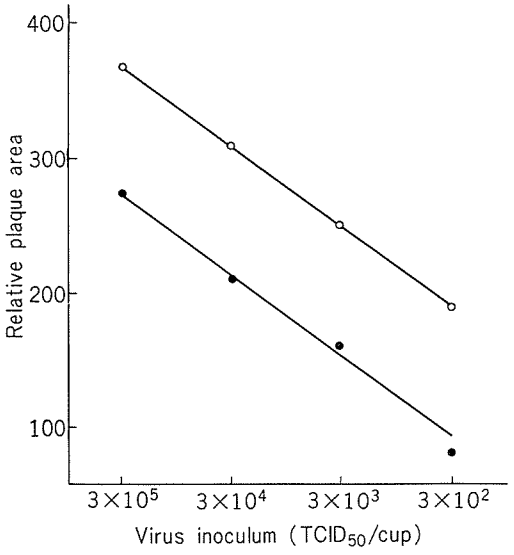

FIGURE 2. *Relationships between virus inoculum size and relative plaque area.* ●——●, *3 days after spot-infection;* ○——○, *4 days after spot-infection.*

These processings were named the " spot-infection technique " and a concentration of 3×10^5 $TCID_{50}$/0.03 ml of virus was regularly used for neutralization tests with spot-infection.

Standard antiserum was prepared in rabbits and diluted to end point neutralization titers of 1: 15,000 to 1: 16,000. When added to the cups with the standard dose of virus the area of plaques was reduced. The reduction was proportional to the concentration of antibody and the method of application of virus and serum to the monolayer agar cup plates. The results of 5 versions of the " spot-neutralization " are shown in Fig. 3. When antibody was placed in agar cups prior to spot-infection, a linear correlation was obtained over a wide range between the relative plaque area and the number of antiserum dilutions. So we

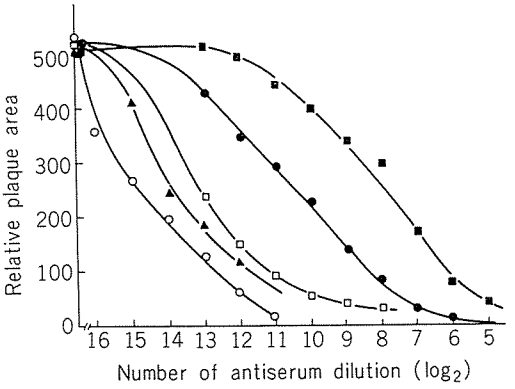

FIGURE 3. *Comparison of sensitivities of 5 different versions of the spot-neutralization system.* ●——●, *0.02 ml of antiserum dilutions were placed in cups and after diffusion for 20 to 30 hr at 37 C under 5% CO_2-air spot-infections were done with 3×10^5 $TCID_{50}$ of virus in 0.03 ml. The dishes were incubated for 4 days and then stained with 2 ml of neutral red agar (1: 7,500);* ■——■, *Spot-infections were carried out 1.5 hr before addition of antiserum dilutions;* ○——○, *Spot-infections were carried out in the cups of the first agar layer containing antiserum dilutions.* □——□, *Spot-infections were carried out for 1.5 hr in the cups of the first agar layer (4.0 ml) and then 2.0 ml of second agar medium containing antiserum dilutions were poured over the first layer;* ▲——▲, *0.2 ml of antiserum dilutions and 0.3 ml of virus antigen were mixed and incubated at 37 C for 3 hr and then overnight at 4 C. 0.05 ml volumes of this mixture were placed in the monolayer agar cups.*

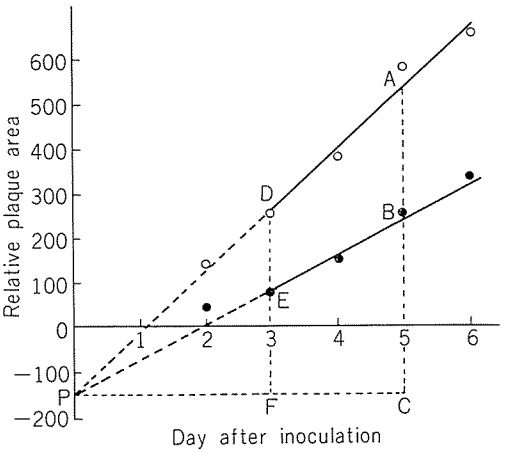

FIGURE 4. *The malleabilities of plaques grown with and without antibody are shown by plotting the relative plaque areas against the number of days after spot-infection.* ○——○, *virus control;* ●——●, *spot-neutralization test with 1: 1024 of antiserum dilutions.*

regarded the procedure as the standard method for the " spot-neutralization test."

The relative plaque areas were also affected by the time after spot-infection, and this time affected the accuracy of the spot-neutralization test. The development of plaques with time in the presence and absence of antibody was carefully observed. The results were plotted as the relative plaque areas against the number of days after spot-infection and gave fairly straight lines converged to a point P (in Fig. 4), inclined with the concentration of antibody. The representative line was drawn and dotted extension lines were added as in Fig. 4. The intersecting points of each line were designated as A, B, C, D, E, F and P. The interpretation of this figure is as follows. $\triangle DPF \backsim \triangle APC$, and $\triangle EPF \backsim \triangle BPC$, and hence $DF/EF = AC/BC = (a_0b_0+150)/(ab+150)$, where, a_0b_0 is the relative plaque area of the virus control and ab is that of the spot-neutralization. These relations indicate that $(a_0b_0+150)/(ab+150)$ should give a constant value against a given dilution of antiserum which is independent of the time allowed for this method. So this value, named the " Ratio of plaque area," was used for neutralizing antibody assay in this method.

$$\text{Ratio of plaque area} = \frac{a_0b_0+150}{ab+150}$$

As described above, the relative plaque area is influenced by the time after infection but the ratio of plaque area is independent of time. So, the relative plaque area (Fig. 3) obtained with spot-neutralization tests after 4 days was converted to the ratio of plaque area (Fig. 5) using the above formula. The scale of the tube end point neutralizing antibody titers is entered in the same figure. It can be seen from the standard curve (Fig. 5) that amounts equivalent to neutralizing antibody titers of as little as 1: 256 can be assayed with the spot-

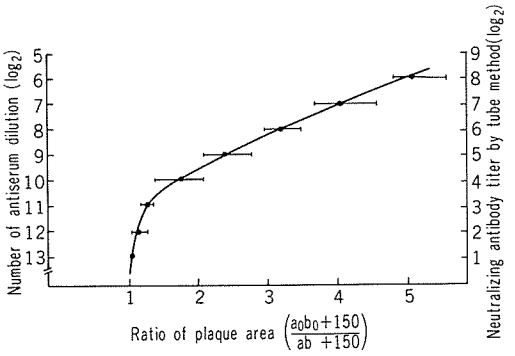

FIGURE 5. *A standard curve for obtaining end point tube neutralization titers from the ratio of plaque area. The results were obtained employing 15 plates (=45 plaques) of each dilution of antiserum.*

neutralization system without dilution of the serum.

Quantities of virus of 3×10^5 to 3×10^2 $TCID_{50}$/0.03 ml were used in the spot-neutralization. As shown in Table 1, the ratio of plaque area did not vary significantly with different doses of virus antigen.

These results show that the neutralizing antibody titers of test sera can be detected and assayed as follows; One drop (0.02 ml) of test serum or control medium is applied to a monolayer agar cup plate and allowed to diffuse overnight in a CO_2-incubator. One drop (0.03 ml) of antigen containing 3×10^5 $TCID_{50}$ of virus is added to the cups. Good circular plaques are stained after a further 4 days. The relative plaque areas are calculated as the products of two diameters measured at right angles to each other with a slide caliper. The relative plaque areas are converted to the ratio of plaque area using formula mentioned before. Then the neutralizing antibody titers of test samples are read from the standard curve in Fig. 5.

To confirm the sensitivity and accuracy of the spot-neutralization test, the results by this method were compared with those by the end point tube dilution method using 30 test sera from healthy adults. A parallel relation was found between the results by the two methods,

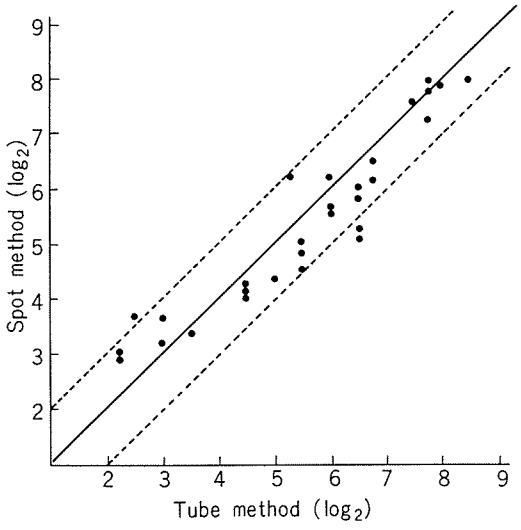

FIGURE 6. *Comparison of the spot-neutralization test with the end point tube dilution method using 30 test sera from healthy adults.*

as shown in Fig. 6. In one serum no neutralizing antibody was detected by either the end point method or this method.

The results in Fig. 6 indicate that the antibody titer of test sera can be assayed without dilution over a wide range of neutralizing titers of from 1: 8 to 1: 256, with the same accuracy as in the end point tube dilution method. Calculation of the antibody titer from the ratio of the plaque areas has given proof of the maximal accuracy to this method (Table 1). The method is based on a similar principle to the neutralization method, namely plaque counts in which the antibody titers are given by the ratio of the number of plaques in dishes inoculated with the virus-antibody mixture and with virus only. In the former the residual virus titer can be expressed as the relative plaque area and in the latter as the plaque count. That is, neither method depends on the "all-or-none law" for assay of virus survival and both take advantage of the so-called "percentage law" (Andrews and Elford, 1933; Dulbecco et al., 1956) in calculating the antibody titer. The results in Table 1 showed that the

TABLE 1. *Effect of the quantity of virus antigen on the ratio of plaque area in the spot-neutralization test*

Virus inoculum ($TCID_{50}$/cup)	Antiserum dilution (log_2)	Two diameters (mm) of plaques Relative plaque area			Mean value of relative plaque area	Ratio of plaque area[b]
3×10^5	V-C[a]	23.2×22.7 526.6	25.5×25.2 642.6	23.6×23.2 547.5	572.2	—
	12	21.7×20.9 453.5	21.8×21.5 468.7	21.9×21.2 464.3	462.2	1.2
	10	16.3×15.8 257.5	15.8×15.7 248.1	15.8×15.3 241.7	249.1	1.8
	9	12.8×12.8 163.8	12.9×12.8 165.1	12.9×12.6 162.5	163.8	2.3
	8	9.4× 9.1 85.5	9.7× 9.1 88.3	9.8× 9.5 93.1	89.0	3.0
3×10^4	V-C[a]	21.9×21.4 468.7	21.5×21.0 451.5	22.0×21.8 479.6	466.6	—
	12	19.2×19.7 378.2	19.4×19.7 382.2	19.2×19.7 378.2	379.5	1.2
	10	13.7×13.8 189.1	13.5×13.8 186.3	13.1×13.0 170.3	181.9	1.9
	9	10.0× 9.5 95.0	10.8×10.9 117.7	10.6×10.7 113.4	108.7	2.4
	8	8.3× 8.3 68.9	7.6× 7.3 55.5	8.0× 8.0 64.0	62.8	2.9
3×10^3	V-C[a]	20.5×20.6 422.3	20.7×20.5 424.4	20.8×20.0 416.0	420.9	—
	12	17.5×17.4 304.5	17.1×17.5 299.3	18.2×17.9 325.8	309.9	1.2
	10	12.7×11.7 148.6	12.4×12.2 151.3	12.3×12.2 150.1	150.0	1.9
	9	9.6× 8.9 85.4	9.0× 8.8 79.2	9.8× 9.3 91.1	85.2	2.4
	8	6.8× 6.2 42.2	7.2× 7.5 54.0	6.8× 6.7 45.6	47.3	2.9
3×10^2	V-C[a]	18.2×18.8 342.2	18.7×18.9 353.4	18.4×17.9 329.4	341.7	—
	12	15.7×16.0 251.2	17.2×16.0 275.2	15.6×16.0 249.6	258.7	1.2
	10	9.3× 8.6 80.0	10.7×10.0 107.0	9.4× 8.8 82.7	89.9	2.0
	9	8.6× 7.7 66.2	8.7× 7.1 61.8	7.8× 7.4 57.7	61.9	2.3
	8	4.5× 3.7 16.7	5.2× 5.0 26.0	4.4× 2.9 12.8	18.5	2.9

a V-C is an abbreviation for virus control where spot-infection was carried out without antibody.
b The ratio of plaque area was calculated from the relative plaque area using formula induced in the text.

antibody titer obtained is independent of the dose of virus, provided the latter is within the range of 3×10^5 to 3×10^2 $TCID_{50}$/cup.

A simple micro neutralization method not involving dilution ($\leqq 1:256$) for 0.02 ml of serum was developed in poliovirus type 1 and Vero cells.

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