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SHORT COMMUNICATION

AN APPLICATION OF PAP (PEROXIDASE-ANTI-PEROXIDASE) STAINING TECHNIQUE FOR THE RAPID TITRATION OF DENGUE VIRUS TYPE 4 INFECTIVITY

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Dengue virus infection is a serious problem in South-east Asian countries. Research of the virus, however, has been hampered by difficulties in the infectivity titration in vitro.

Several investigators have reported on assay methods of the infectivity of dengue viruses by plaque formation (Schulze and Schlesinger, 1963; Russel et al., 1967; Fujita et al., 1975). In plaque titration, however, a longer incubation time is necessary to obtain the results; usually about one week. Igarashi and Mantani (1974) successfully employed the indirect fluorescent antibody (FA) technique for the rapid titration of dengue type 4 virus (D4) by counting the fluorescent foci. Research has been much accelerated by their method as titration is completed within two days after inoculation.

Recently the immuno-peroxidase technique has been introduced and widely employed to identify virus antigens in infected cells (Wicker and Avrameas, 1969; Kurstak, 1971; Ubertini et al., 1971; Shabo et al., 1972; Dubois-Dalcq and Barbosa, 1973; Herrmann et al., 1974; Benjamin, 1974; Miller et al., 1974; Gerna et al., 1976). The technique has also been applied for the quantitative assay of influenza virus infectivity (Hahon et al., 1975). This technique offers a number of advantages in that the staining is stable and the specimens can be examined with an ordinary light microscope, while in the FA technique the stained cells easily fade out, even during examination, and observation must be performed in the dark field of a fluorescence microscope.

Sutmoller and Cowan (1974) compared three immuno-peroxidase techniques (direct, indirect and peroxidase-anti-peroxidase [PAP] methods) for the detection of Foot-and-Mouth disease virus and recommended the PAP technique as the most suitable method in terms of background staining and sensitivity. In our own preliminary experiments the PAP technique was the most satisfactory for the assay of dengue virus infectivity.

As we are concerned with establishing a rapid and ubiquitous titration method for all types of dengue virus, we applied the PAP technique in comparison with the indirect FA technique for assaying D4 virus infectivity.

Throughout the experiments the methods of Sternberger et al. (1970) and Dougherty et al. (1972) were essentially used with slight modifications in detail.

D4 virus was grown in suckling mouse brains. Ten percent homogenates of the infected brains in Eagle's minimum essential medium (MEM), containing 0.25% bovine plasma albumin (Fraction V, Armour Pharmaceutical Co., Chicago, Ill., U.S.A.), were centrifuged at 10,000 rpm for 30 min. The supernatant was stored at −70 C as the stock virus.

Antisera to D4 virus were obtained in rabbits by an intravenous injection of 3 ml of the stock virus, followed by 7 times intramuscular injection of 5 ml each of the stock virus at weekly intervals. The rabbits were sacrificed one month after the last injection. The average HI titer of sera was 640 against 4 U of the antigen.

Antisera to peroxidase (PO) were obtained in rabbits by two times intramuscular injectoin with 10 mg of soluble PO (horseradish peroxidase, type VI, RZ=2.65, Sigma Chemicals, St. Louis, U.S.A.) in complete Freund's adjuvant at two weeks intervals. The rabbits were boostered with 2 mg soluble PO in saline subcutaneously 2 weeks after the 2nd PO-adjuvant injection. The same level of specific antibody was found in sera obtained from partial bleedings at the 1, 2, 3, and 4th week after

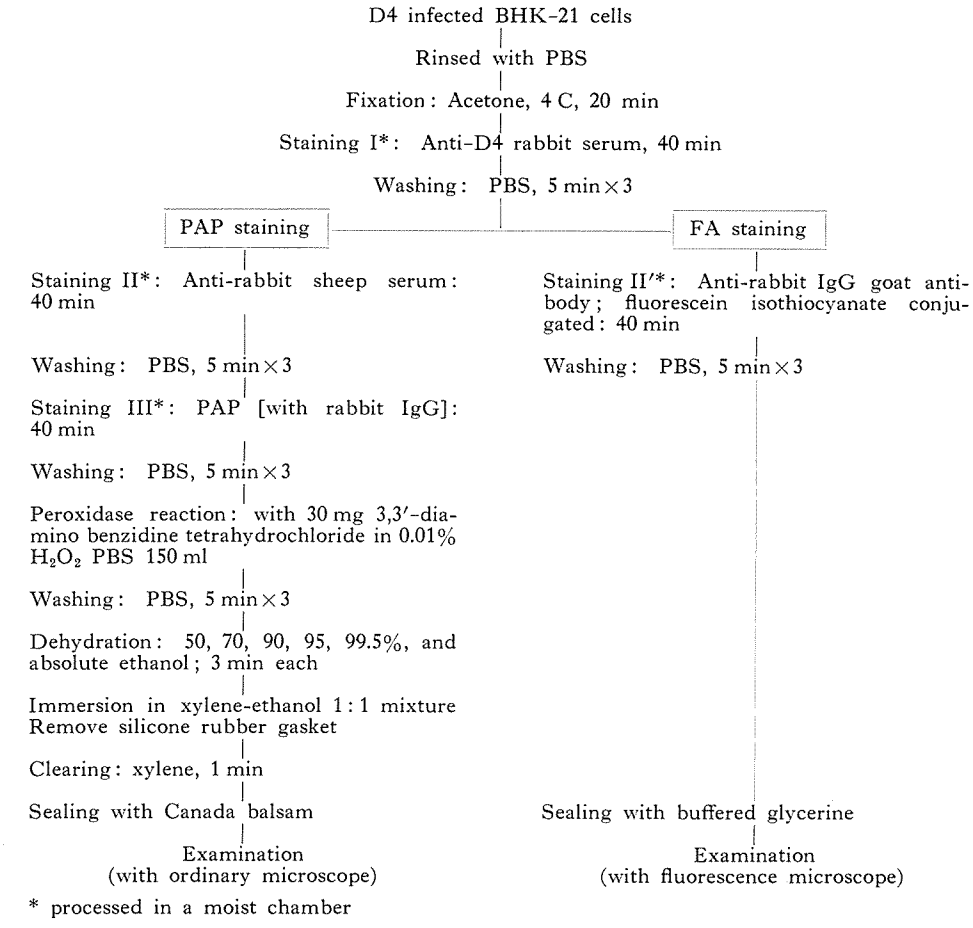

FIGURE 1. Staining procedures used (PAP and indirect FA techniques)

the booster injection, by micro-Ouchterlony method.

Sheep antisera to rabbit IgG were kindly supplied by Kanonji Institute of The Research Foundation for Microbial Diseases, Osaka University.

The titer of anti-PO antibody was estimated by the quantitative precipitation reaction (Dougherty et al., 1972) and the soluble PAP complex was prepared by the method of Sternberger (1970).

The procedures for cell preparation were as follows: about 1×10^5 of BHK-21 cells per well were grown in Lab-Tek 8 chamber tissue culture slides (Miles, Ill., U.S.A.) in a CO_2 incubator at 37 C. After incubation for about 24 hr, the cell monolayers of each well were inoculated with 0.1 ml of serially diluted D4 stock virus. After 2 hours adsorption the cells were rinsed with 0.5 ml of Hanks' balanced salt solution and then covered with 0.4 ml of MEM added with 2% fetal calf serum and incubated again in a CO_2 incubator at 37 C.

Two days after the inoculation, the fluid phase was removed and the cells in the wells were washed with phosphate buffered saline (PBS, pH 7.4) and fixed with pre-cooled acetone at 4 C for 20 min.

Figure 1 shows the staining procedures used for both PAP and FA techniques. All the procedures were carried out at room temperature. Three immunological stainings (with anti-D4 virus rabbit serum diluted to 1/150, anti-rabbit IgG sheep serum diluted to 1/150, anti-rabbit IgG sheep serum diluted to 1/50, and PAP complex [with rabbit IgG] diluted to 1/10) of PAP technique and two immunological stainings (with anti-D4 virus rabbit serum diluted to 1/100 and fluorescein isothiocyanate conjugated anti-rabbit IgG goat serum (Hyland Corp., Calif., U.S.A.) diluted to 1/70) of indirect FA technique were applied for 40 min each in a moist chamber. After each step, the slides were rinsed with 3 changes of PBS, 5 min each.

In the final step of PAP technique the pero-

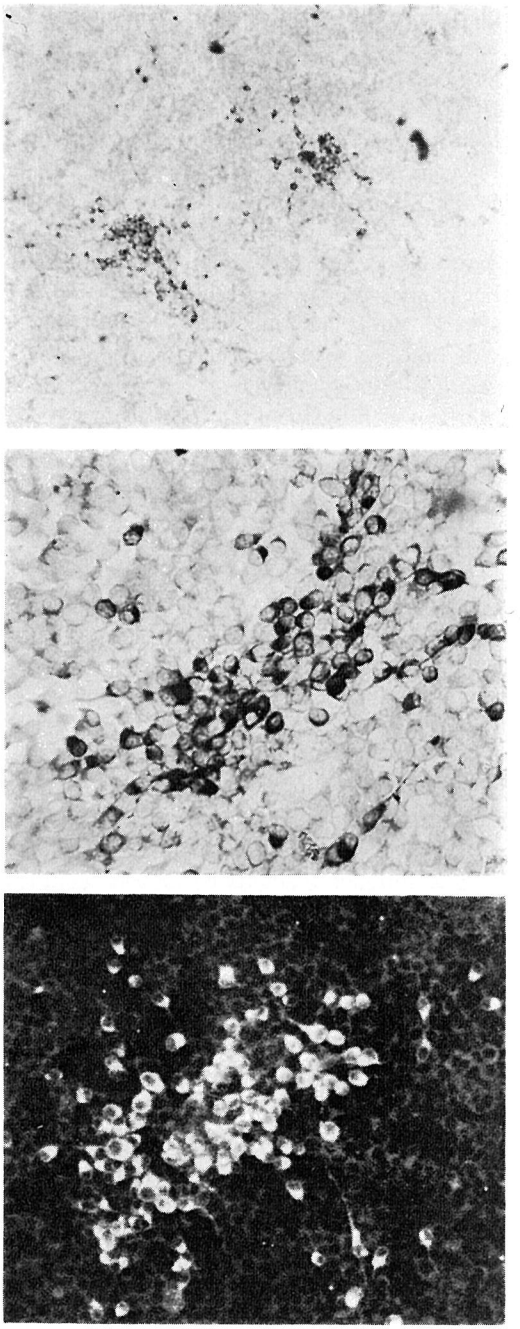

FIGURE 2. Foci in D4 infected BHK-21 cells stained by PAP technique (A and B), and indirect FA technique (C). D4 virus antigen is clearly shown in cytoplasm.

xidase reaction was developed following the method of Graham and Karnovsky (1966). The cells were immersed for 5 min in 150 ml PBS containing 0.01% H_2O_2 and 30 mg of 3–3′-diaminobenzidine tetrahydrochloride. Then the cells were washed in PBS, dehydrated through a graded ethanol series, cleared in xylene, and sealed with Canada balsam. Observations were made, on occasion, under ordinary microscope.

As for the FA technique, the cells were sealed with buffered glycerin after the staining and examined immediately in the dark field of an interference filter type fluorescence microscope with a halogen lamp light source, to minimize the fading out of the staining.

Examples of foci in BHK-21 D4 virus system stained by both methods are shown in Fig. 2. The foci were proved to be specific to the D4 virus infection, by the examination of such controls as the uninfected cells with the complete system, the normal rabbit serum used for staining step I and the deletion of each step from the three staining steps.

Figure 3 shows a linear relationship between logarithms of the virus dilution and the numbers of foci per well stained by the PAP and FA techniques. The results obtained by both techniques seem to be almost identical. This demonstrates that both techniques are equally valuable for the assay of this virus and that the FA technique can be replaced by the PAP technique.

Our preliminary experiments on other types of the virus, employing each homologous antiserum for staining step 1, indicated that the foci of type 2 could be counted 2 days after inoculation and stained as strong as D4 but the staining was faint in case of type 1 and 3 virus infected cells compared with D4 even 3 days after inoculation. This may reflect the difference in growth characteristics among virus types.

As the examination is performed in bright field and the visible field is wider, the counting of the foci stained by the PAP technique is easier and more accurate compared to FA foci

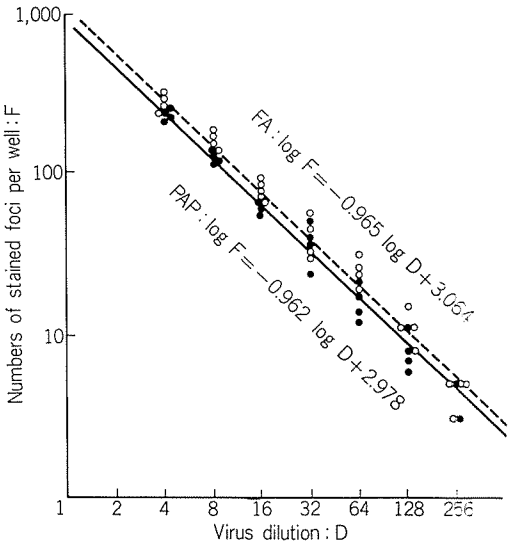

FIGURE 3. Relationship between the D4 virus dilution and numbers of foci per well stained by PAP (●) and indirect FA (○) techniques. The least squares regression lines obtained by both PAP (———) and FA (– – – –) techniques are shown.

counting in which a dark field is necessary and the observable field is limited and narrow. Moreover, it is very convenient, as has been claimed by other workers, especially when a number of specimens are prepared at the same time, that the PAP stained slides can be preserved with no change until later observations.

The preparation of PAP was rather easy and when stored at 4 C it has been stable at least for six months. Hyperimmune sera, however, are essential to obtain a qualified PAP complex. The excessive residual aliquots of PAP used for the staining can be re-used several times if they are recovered from the slides after staining.

Though the PAP technique includes more steps than the FA technique and may seem tedious, the PAP technique has several advantages over the FA technique once the conditions are established and is worthy of practical routine use.

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