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SHORT COMMUNICATION

APPLICATION OF THE PAP (PEROXIDASE-ANTI-PEROXIDASE) STAINING TECHNIQUE FOR THE RAPID TITRATION OF MUMPS VIRUS INFECTIVITY

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Application of the PAP technique for infectivity assay of mumps virus provides a fast, reproducible, and convenient assay system, which is better than other methods reported previously.

The infectivity of mumps virus can be determined by plaque formation (Hotchin et al., 1960; Frothingham and Granoff, 1961; Sato et al., 1978), cytopathic change (Buynak and Hillman, 1966; Henny et al., 1970) and hemadsorption (Buynak and Hillman, 1966; Yamanishi et al., 1970), but 4–10 days are required to obtain results by these methods. Later the plaque method was improved by Sato (1979) to be more economical and to take only 3 days. However, this improved method is still complex and is only applicable to Vero-adapted strains, when Vero cells are used as indicator cells. Recently, the immunofluorescent technique (Bjorvatn and Wolontis, 1972; Lennette et al., 1975) and immunoperoxidase method (Benjamin and Ray, 1974) have been employed for rapid diagnosis of mumps virus in tissue culture. With these methods, a single cell containing virus antigens can be marked. A comparative study of the two methods demonstrated their comparable sensitivity (Hahon et al., 1975) for virus quantification. However, the immunoperoxidase method has a number of advantages over the immunofluorescence method, including stability of the stain and clearer detection of viral antigens under a light microscope. There are three methods for the immunoperoxidase technique; direct, indirect, and peroxidase-anti-peroxidase (PAP) methods, of which the third is the best in terms of staining and sensitivity (Sutmoller and Cowan, 1974). Okuno et al. (1977; 1978) used the PAP staining technique for virus infectivity titration and demonstrated the advantages of the method for this purpose. This method is fast and easy and when Lab-Tek 8 chambers (Milles Lab., Ill.) are used, the method is economical with respect to cell numbers, medium volumes, and glassware space. Thus, we applied the PAP staining technique for titration of mumps virus infectivity and found

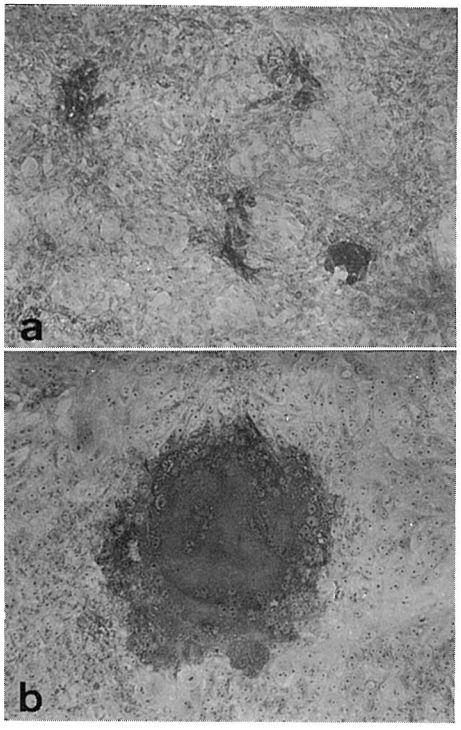

FIGURE 1. Foci formed by mumps virus infection on Vero cells, stained by PAP technique (46 h after virus inoculation). Scale: ×150

a. Urabe strain, b. Enders strain.

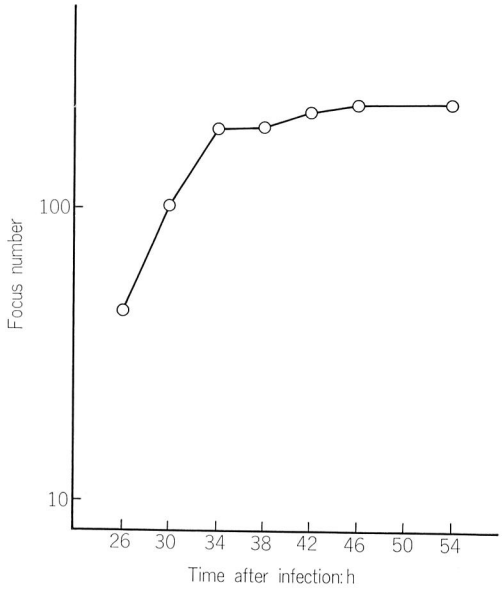

FIGURE 2. Effect of incubation time on focus formation by Urabe strain.

After incubation for the times indicated, virus-inoculated Vero cells were washed, fixed, and stained with PAP method. Then stained foci were counted.

that it was better than other methods.

Amnion-adapted mumps virus, Urabe strain, which was kindly given by Dr. K. Yamanishi (this Institute), was grown in the amniotic cavity of chick embryos. This infected amniotic fluid was stored at −120 C as virus stock. Antiserum to the virus was raised in rabbits by three intramuscular injections of virions purified by sucrose gradient centrifugation (Hosaka and Hosokawa, 1977), together with complete Freund adjuvant (DIFCO Lab., Michigan). The hemagglutination inhibition titer of the antiserum was 640. Goat IgG to rabbit IgG was obtained from the Research Foundation for Microbial Diseases of Osaka Univ. PAP conjugated with rabbit IgG was from Cappel Lab. (Penn).

Vero cells or chick embryo fibroblasts (CEF) were propagated to monolayers in Lab-Tek 8 chambers and incubated with 0.1 ml/well of serially diluted mumps virus. After 1 h, unadsorbed virus was removed and the cells were covered with 0.3 ml of MEM containing 2% fetal calf serum (GIBCO Lab., N.Y.) and 1% tragacanth gum (Wako Chem. Indust., Osaka), and incubated in a CO_2 incubator at 37 C. Then the fluid phase was removed and the cells were fixed with precooled acetone at 4 C for 20 min. The cells were sequentially stained with 1/500 dilution of anti-mumps virus rabbit serum, 1/100 dilution of anti-rabbit IgG/goat IgG, and 1/1000 dilution of PAP complex conjugated with rabbit IgG. Each step was done at room temperature for 40 min in a moist chamber, and the cells were washed with PBS between each step. Thereafter the peroxidase reaction was developed on the slides by the method of Okuno et al. (1977), and the stained foci were counted.

The egg-adapted Urabe strain was reported

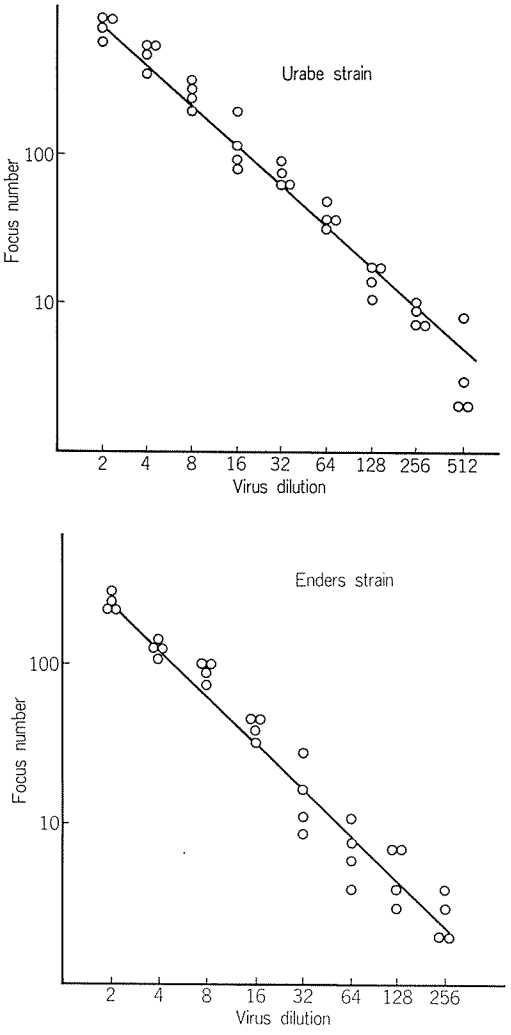

FIGURE 3. Relationship between virus dilution and focus number.
a. Urabe strain, b. Enders strain.

to grow well in CEF (Naruse et al., 1981), but it grows poorly in Vero cells, as shown later. Therefore we first examined which cells were better as indicator cells for PAP-focus formation by mumps virus. Figure la shows the PAP-foci on Vero cells. These foci were found to be clearer and more easily distinguished from the background of unstained cells than those on CEF. One possible reason for this was that the anti-virus serum used was hyperimmune serum prepared with virions grown in the amniotic cavity of chick embryos and might contain some antibodies to host components. Another advantage of use of Vero cells was that the cells are an established line and usually become firmly attached to the dish wall. Therefore, we used Vero cells as indicator cells. The variety of size of the foci observed, as shown in Fig. la, may reflect heterogeneity in the growth phase of each virus.

Next, we determined the optimal incubation time for focus formation. Figure 2 shows the effect of the incubation time on focus formation. The number of foci reached a plateau at 42 h after virus inoculation. Therefore, we used an incubation period of 46 h for focus formation in the assay.

Figure 3a shows the linear relation obtained between the dilution of the Urabe strain and the number of foci. This finding indicated that the PAP technique is useful for infectivity assay of the Urabe strain. One HA was found to correspond to about 10^6 foci, which is comparable with the best results with other assay systems.

We apllied this technique for mumps virus, Enders stain, which had been passed in Vero cells and was kindly given by Mr. Takamizawa (Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka Univ.). Figure 1b shows a focus of Enders strain on Vero cells: it is smooth and larger than those of the Urabe strain. The focus size tended to increase with the incubation time, but the focus number remained unchanged after 40 h incubation. Thus, this PAP technique was also suitable for Enders strain. Only problem with Enders strain was that since the focus size was big, the maximum countable focus number was only 200-300/well.

Using this technique, we determined the growth curve of the Urabe strain in Vero cells (Fig. 4). The virus titer in the fluid remained almost constant ($10^{4.0}$—$10^{5.0}$ FFU/ml) during 4 days culture, but the titer of cell-associated virus reached a maximum ($10^{3.9}$ FFU/ml) at

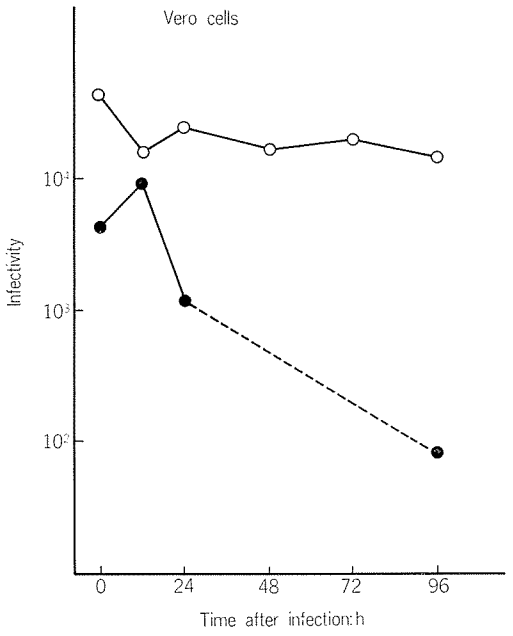

FIGURE 4. Growth curve of Urabe strain in Vero cells.

Vero cells in Falcon dishes (3001) were infected with Urabe strain at moi 10. The cells were washed 1 h later and incubated in 2 ml of MEM containing 2% feta calf serum. At intervals, culture fluids and cells were harvested and stocked at −120 C until infectivity assay. ○; infectivity titer (focus forming units=FFU/ml) in the fluid, ●; cell-associated titer.

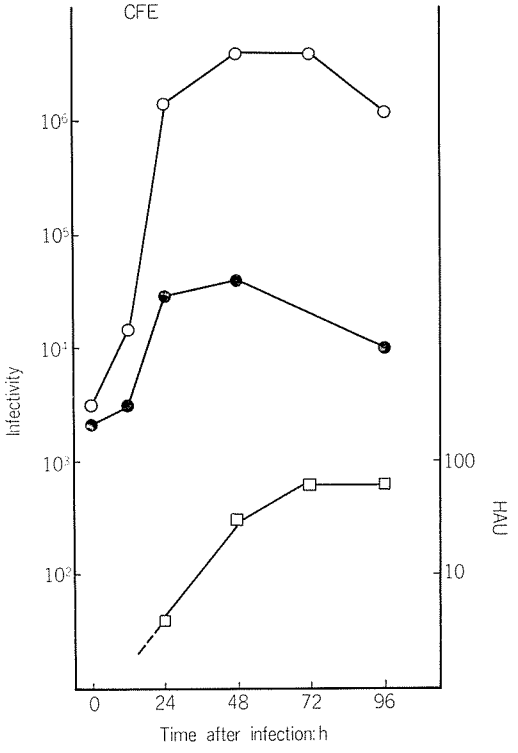

FIGURE 5. Growth curve of Urabe strain in CEF. Infection with the virus was as for Vero cells. □; HAU (Hemagglutination units). The other symbols are as in Fig. 4.

12 h after virus inoculation, and then decreased. These findings suggested that virus of the Urabe strain matured poorly in Vero cells, although virus antigen synthesis occurred unequivocally. On the contrary, the Urabe strain was found to grow well in CEF. The virus titer in the fluid reached a maximum 48 h after inoculation, and that of cell-associated virus reached also a maximum at about the same time. Only in this system was released hemagglutinin detected, and its production was seen after that of new infectious particles. The growth curve was essentially similar to that reported by Naruse et al. (1981), who measued infectivity by the hemadsorption method (Yamanishi et al., 1973).

Thus, the present PAP-focus method provides a fast and convenient assay system for mumps virus infectivity.

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REFERENCES

Benjamin, D. R., Ray, C. C. 1974. Use of immunoperoxidase for the rapid identification of human myxoviruses and paramyxoviruses in tissue culture. Appl. Microbiol. 28: 47–51.

Bjorvatn, B., Wolontis, S. 1972. Laboratory diagnosis of mumps by immunofluorescence on experimentally inoculated cell cultures. Scand. J. Infect. Dis. 4: 105–108.

Buynak, E. B., Hillman, M. R. 1966. Live attenuated mumps virus vaccine. I. Vaccine development. Proc. Soc. Exp. Biol. Med. 123: 768–775.

Frothingham, T. E., Granoff, A. 1961. Plaque formation with mumps virus. Virology 15: 213–214.

Hahon, N., Simpson. J, Eckert, H. L. 1975. Assessment of virus infectivity by the immunofluorescence and immunoperoxidase techniques. J. Clin. Microbiol. 1: 324–329.

Hosaka, Y., Hosokawa, Y. 1977. Purification of Sendai virions with glutaraldehyde-treated red blood cells. Intervirology 8: 1–17.

Hotchin, J. E., Seibel, R., Benson, L. M. 1960. Location of noncytopathic myxovirus palques by hemadsorption. Virology 10: 275–280.

Kenny, M. T., Albright, K. L., Sanderson, R. P. 1970. Microneutralization test for the determination of mumps antibody in Vero cells. Appl. Microbiol. 20: 371–373.

Lennette, D. A., Emmons, R. W., Lennette, E. H. 1975. Rapid diagnosis of mumps virus infections immunofluorescence methods. J. Clin. Microbiol. 2: 81–84.

Naruse, H., Nagai, Y., Yoshida, T., Hamaguchi, M., Matsumoto, T., Isomura, S., Suzuki, S. 1981. The polypeptides of mumps virus and their synthesis in infected chick embryo cells. Virology 112: 119–130.

Okuno, Y., Igarashi, A., Fukai, K. 1978. Neutralization tests for dengue and Japanese encephalitis viruses by the focus reduction method using peroxidase anti-peroxidase staining. Biken. J. 21: 137–147.

Okuno, Y., Sasao, F., Fukunaga, T., Fukai, K. 1977. An application of PAP (peroxidase-anti-peroxidase) staining technique for the rapid titration of dengue virus type 4 infectivity. Biken J. 20: 29–33.

Sato, H., Albrecht, P., Hicks, J. T., Meyer, B. C., Ennis, F. A. 1978. Sensitive neutralization test for virus antibody I. Mumps antibody. Arch. Virol. 58: 301–311.

Sato, H., Albrecht, P., Krugman, S., Ennis, F. A. 1979. Sensitive neutralization test for rubella antibody. J. Clin. Microbiol. 9: 259–265.

Sutmoller, P., Cowan, K. M. 1974. The detection of Foot-and-Mouth disease virus antigens in infected cell cultures by immuno-peroxidase techniques. J. Gen. Virol. 22: 287–291.

Yamanishi, K., Takahashi, M., Ueda, S., Minekawa, Y., Ogino, T., Suzuki, N., Okuno, Y., Kan, T. 1973. Studies on live mumps virus vaccine. V. Development of a new mumps vaccine "AM9" by plaque cloning. Biken J. 16: 161–166.