CHAPTER III

MATERIALS AND METHODS

Materials

1. Cell Culture

In the virological laboratory there are several cell lines that were used for herpes simplex virus propagation. In this study, we employed two cell lines which were described previously (27,47). Cervical carcinoma (HeLa) cells and African green monkey kidney (Vero) cells were kindly supplied by Virus Research Instituted, Department of Medical Sciences, Ministry of Public Health, Thailand.

2. Virus

Herpes simplex virus type 2 (strain LB) was originally obtained from University of Illinois at the Health Sciences Center, Chicago, Illinois, U.S.A.

3. Tested Samples

Ninety-two samples of human sera were collected from the patients attending at the Antenatal Care Unit of Chulalongkorn Hospital. After centrifugation, these sera were separated, aliquoted, and stored at -20 ^{o}C for later use.

4. ELISA kit

Commercial ELISA kit (Behringwerke, W. Germany) was used for a standard test in detecting HSV-antibody.

4.1 Test-plate

Test-plate composed with 12 x 8 reaction wells, they were devided into 12 x 4 sets of the test. Each set was arranged so that the upper row in each set being coated with test-antigen and the lower with control antigen. Test antigen and control antigen were obtained from HSV-infected HeLa cells and uninfected cells, respectively. The antigens were inactivated before coating.

4.2 Test-sample

Test-sera were diluted 1:44 in determination of the immune status (IgG). Samples should not be preinactivated (56 °C for 30 min) before use.

4.3 Conjugate

Anti-Human IgG/AP Conjugate (γ - chain-specific) for "Enzygnost" were produced by the coupling of alkaline phosphatase (AP) to highly avid antibodies from the rabbit.

Methods

1. Preparation of antigen for ELISA

1.1 Preparation of cell culture

The procedure used for two cell cultures propagation were following (47,88). The medium in cell-monolayer was withdrawn and discarded. The monolayer was then washed with phosphate buffer saline (PBS, free from Ca^{++}, Mg^{++}) to remove trace of serum. After which it was detached with trypsin-versene (TV) solution by incubating at room temperature until the cell rounded up and the TV-solution was removed. The cell suspension was shaken off and diluted to 1.5×10^5 cells / ml with growth - medium containing Minimal Essential Medium (MEM, GIBCO, Grand Island, New York, U.S.A.) plus 10 % fetal bovine serum (FBS) (Flow laboratories, North Ryde, Australia). Finally, cell suspension (1.5×10^5 cells/ml) was seeded onto new tissue culture bottles and further incubated at $37^{o}C$ until the confluent cell - monolayer was obtained, at which point the cells should be passaged (Fig.1)

1.2 Propagation of stock virus

The propagation of HSV-2 from stock seed-virus was carried out by the following procedure. A volume of 10 ml of single cell suspension (3.5×10^5 cell/ml) in the growth medium was seeded onto a tissue culture bottle (8 OZ.) and incubated at $37^{o}C$ for 24 h. After this time,

the medium on the confluent monolayer of cells was withdrawn and discarded. The monolayer was washed twice with PBS to remove trace of serum. Two milliliters of stock HSV-2 diluted 1:10 in maintenance medium was inoculated onto the monolayer and allowed to adsorb at 37°C for 2 h. During the incubation period, the culture bottles were rolled every 30 min. At the end of the incubation period, 8 ml of maintenance-medium was added onto the infected monolayer and further incubated at 37°C for 24 h, at which time normally 3+ cytopathic effect (CPE) was formed (Fig.2). The infected cells were disrupted by three cycles of freezing and thawing. The cell debris was removed by centrifugation at 2,500 r.p.m. for 10 min in 4°C.

Pools of stock virus, prepared in either HeLa or Vero cells were divided in small aliquots and kept at -70°C until used. Titer of the virus was determined by plaque forming assay (89,90).

1.3 Titration of stock virus

Plaque forming assay is the measurement of infectivity of the virus (titer). In this method, the number of plaques equal to the number of infectious units. Titrations of HSV-2 were performed on HeLa and Vero cells, using Linbro - 12 wells titerplate (Flow Laboratories, Hamden, Connecticut, U.S.A.). An inoculum of 0.1 ml was added onto the cell monolayers and the viruses permitted

to adsorb at 37 °C for 2h. Finally, infected cell monolayers were overlayered with MEM containing 0.5 % agarose and further incubated at 37 °C for 3 days. Number of plaques was counted after the virus was inactivated by 10 % formalin and stained with 0.5 % crystal violet. The virus concentration (titer) was expressed as number of plaque forming unit per ml (PFU) (Fig.3).

1.4 Virus inactivation Assays

The virus was inactivated in order to destroy infectivity before use (91). The procedures of viral inactivation were carried out in three following studies :

1.4.1 UV-inactivation : UV inactivated HSV was prepared by exposing 3 ml of the stock-virus, on an opened 90 mm glass - petridish, to a germicidal lamp (G 30T8, Sylvania Electric Products,Danver,Mass., U.S.A.). The exposure was allowed at a distance of 1 ft in the duration of 10 - 75 min.

1.4.2 Formalin-inactivation : Formalin inactivated HSV was prepared by adding formalin-solution in to stock-virus, adjusted to have final concentration of 0.1 to 1% formalin, and incubated at room temperature for 3 h.

1.4.3 Heat-inactivation : Heat inactivated HSV was performed by incubating 3 ml of the stock-virus in a water bath having temperatures ranging from 30 °C to 60 °C.

The infectivity of the inactivated-HSV was determined by inoculate the inactivated virus into cell culture and observed appearing of CPE, and also by the plaque assay titration.

1.5 Selection of antigen

1.5.1 HSV antigen-preparations in MEM and PBS

Vero cells were grown and then washed with PBS as previously described. After which 2 ml of HSV-2 (1.3×10^7 PFU/ml) diluted 1:20 in MEM without serum was inoculated and adsorbed at 37 °C for 2 h. During the incubation period, the cultured bottles were rolled every 30 min. At the end of the adsorption, 4 ml of MEM without serum was added and further incubated at 37 °C. They were terminated at the time showing CPE 3+ to 4+. Two procedures for antigen collections were as follow.

1.5.1.1 Collection of viral antigens from serum-free medium and infected cells : After cell cultures showed 3+ to 4+ CPE, the culture bottles were kept at -70 °C. The virus were free from the cells by three cycles of freezing and thawing. The cell debris was removed by centrifugation at 2,500 r.p.m. for 10 min at 4 °C. The supernate containing the antigen for ELISA was kept at -20 °C until used.

1.5.1.2 Collection of viral antigens from PBS washed infected cells : Herpes simplex virus was

propagation as previously described. When cell culture showed 3+ to 4+ CPE, the infected cells were detached by scraping and collected by centrifugation (1,500 r.p.m.) for 10 min at 4°C and washed twice with PBS. The pellet was then diluted in PBS to make final of 10 % cell suspension. The infected cell suspension was disrupted by three cycles of freezing and thawing. The cell debris was removed by centrifugation at 3,000 r.p.m.for 30 min at 4°C. Finally, the supernate was collected as a source of viral antigen for ELISA.

1.5.2 HSV antigen-preparation in HeLa and Vero cells.

Results from the preceding experiment suggested the use of PBS for antigen preparation. HSV was propagated in HeLa and Vero cells as previously described in 1.5.1.2

These viral antigens were inactivated by heat at 50°C 1 h. They were kept frozen at -20°C and sonicated before use. Control antigen obtained from uninfected cells was treated as previously described. Concentration of the protein in the crude antigens preparation was determined by Lowry's method (92).

These antigens were comparatively used for detecting HSV antibody. The ELISA procedure was modified by Voller et al. and Hampar et al. (8,85). Each antigen

was diluted to 40 µg/ml in the coating buffer, pH 9.6, and were adsorbed to wells (100 µl/well) of flat - bottom polystyrene microtiter plate (Nunc Inc., Roskilde, Denmark). Lower row, control antigens were processed as the same way. Adsorption was carried out overnight at 4 °C. After washing, 50 µl of HSV-positive serum diluted in PBS-Tween-albumin was added to the wells in duplicate. The plate was incubated at 37 °C for 90 min in a moist chamber. Each well was washed five times with PBS-Tween. After which 50 µl of peroxidase conjugated antihuman IgG (Dako, Glostrup, Denmark) diluted 1:200 in PBS-Tween-albumin was added into each well and was further incubated at 37 °C for 1 h. The wells were washed five times as above, and 100 µl of O-phenylenediamine substrate solution was added to each well. Finally, the plate was incubated in the dark at room temperature for 30 min. The reaction was stopped by adding 50 µl of 4 N H_2SO_4. The plate was measured at 492 nm with ELISA -microtiter plate reader.

The results were evaluated for selection of the better antigen. Our preliminary data indicated that HSV antigen which prepared in HeLa cell and suspended in PBS was better than the other one and was used for the further experiments.

1.6 Verification of viral antigen

Several batches of antigen preparation were pooled and processed. The sample of HSV-2 infected cells

of each batch were verified with a monoclonal antibody to HSV-2 by indirect immunofluorescent antibody technic (IFA) (71,93).

HSV-2 monoclonal antibody (2D-clone, which was kindly supplied by National Institute of Health, Thailand) was applied onto smear of the HSV-2 infected cells and allowed to react in the moist chamber at 37 °C for 30 min. After the incubation period, the slides were extensively rinsed with three changes of PBS. Fluorescein-conjugated goat - anti - mouse -IgG (Cappel laboratories, Cochranville, PA, U.S.A.) was added on the smear and incubated for 1 h at 37 °C in a moist chamber. The slides were then washed with PBS as described above, and counterstained with Evans blue for 1 min. Finally, the slides were dried and mounted with buffered glycerol and examined under a fluorescent microscope. HSV-2 infected cells fluoresced with 3+ to 4+ intensity (Fig.4) while the uninfected cells showed no fluorescence (Fig.5).

2. Determination of Factors Affecting the ELISA System

The indirect ELISA was used in this study. In order to establish the assay, checker board titration was carried out to determine the optimal conditions of the reagents in this assay (8,85,94,95).

2.1 Determination for optimal concentration of the reagent used

- Diluted HSV-2 antigen in carbonate - bicarbonate buffer (coating buffer), pH 9.6 to give the final concentrations of 20,40,80, and 160 µg of protein/ml.

- Added 100 µl of 20 µg/ml of HSV-2 antigen into wells of a polystyrene microplate (1st, 2nd, and 3rd columns; A,C,E,and G rows) (Fig.6). Control antigen was similarly diluted and added to rows B,D,F,and H. The antigen concentrations of 40, 80, and 160 µg/ml were added into wells 4th - 6th, 7th - 9th, and 10th - 12th, respectively by the same way.

- Covered the plate and incubated at 4°C overnight.

- Shaken off the contents, the wells were refilled with PBS-Tween, allowed to stand for 10 min, shaken off, and repeated this washing process twice.

- Diluted 1:50, 1:100, and 1:200 of HSV positive serum (Behringwerke AG, Marburg, W.Germany) in PBS-Tween albumin.

- Added 50 µl of each dilution into wells of the 1st, 2nd, and 3rd columns, respectively. This procedure was identically repeated in the other sections. The plate was incubated at 37°C for 90 min in a moist chamber.

- Washed plate 5 times with PBS-Tween.

- Diluted 1:1,200, 1:2,400, 1:4,800, and 1:9,600 of peroxidase-conjugated rabbit immunoglobulins to human IgG in PBS-Tween-albumin.

- Added 50 µl of each dilution into wells of A - B rows, E - F rows, and G - H rows,respectively and incubated at 37 °C for 1 h.

- Washed plate 5 times as previously described.

- Added 100 µl of substrate solution (O-phenylenediamine in phosphate-citrate buffer, pH 5 and 30% H_2O_2) for 30 min.

- Stopped reaction by 50 µl of 4 N H_2SO_4 and color developed was determined by a ELISA-microtiter plate reader at an absorbance of 492 nm.

For determination of optimal condition in this study, the pools of human sera (Antenatal Care Unit of Chulalongkorn Hospital) with high titer antibodies (1:32) to HSV by complement fixation test and positive antibodies to HSV by indirect immunofluorescent antibody technic were used as HSV-strong-positive-control-antibody, because we found that HSV-positive commercial serum was a weak-positive. Pooled serum was aliquoted and stored at - 20 °C. HSV-negative control antibody, the normal human serum with negative-antibody to HSV, examined by the same method, was selected from a graduate - student of Inter - Departments of Medical Microbiology, Chulalongkorn University. These positive and negative control sera were determined by the procedure as described

previously.

Optimal conditions should be the highest dilution of capture antigen antibody and conjugate which gave the highest positive value and the lowest negative value.

2.2 Temperature and time-course for coating antigen on the plate

To determine the optimal temperature and time-course for HSV-antigen in coating on plates, 100 µl of HSV-antigen was diluted to give final concentration of 80 µg of protien per ml in coating buffer, pH 9.6 and pipetted in duplicate into wells of 5 strips (Microwell Module, Nunc, Roskilde, Denmark). Control antigen was added onto the wells next to this antigen. Each of strips was incubated at 4 °C overnight, room temperature overnight, 37 °C for 2 h and kept at 4 °C overnight, 37 °C for 6 h and kept at 4 °C overnight, and 37 °C overnight. The appropiate dilution of each reagent (from checkerboard titration) was added, and then processed as in the screening assay.

2.3 Determination of time-course for :

2.3.1 HSV-antigen and antibody reaction

Fifty microliters of positive-antibody to HSV diluted 1:100 with PBS-Tween-albumin was pipetted into duplicate wells of 4 strips, precoated with 80 µg/ml

of HSV and control antigen as previously described. Negative-antibody to HSV was prepared in the same manner. The strips were incubated at 37 °C for periods varying from 30 to 120 min (30, 60, 90, and 120 minutes), and processed as in the screening assay.

2.3.2 Peroxidase conjugated antiserum of rabbit immunoglobulin to human IgG reaction

Following antigen and antibody reaction, 50 µl of 1:2,400 antihuman-IgG peroxidase-conjugate was added to each well, and the strips were further incubated at 37 °C for periods varying from 15 to 120 min(15, 30, 60, 90, and 120 minutes). The plates were then processed as in the screening assay.

2.3.3 Color development

One hundred microliters of O-phenylenediamine substrate solution was added to each well after antibody and conjugate reaction. The strips were incubated at room temperature in the dark for periods varying from 10 to 60 min (10, 20, 30, 40, 50, and 60 minutes). At the end of color developing, 50 µl of 4 N H_2SO_4 was added into each well to stop the reaction.

3. Standardization of the ELISA test.

3.1 Precision study

A within-plate precision was determined by

using three sera; HSV-strong-positive control, HSV-positive commercial antibody, and negative control. Precision for each serum was calculated by running 20 replicates in one microtiter plate.

A between-plate precission (reproducibility) was determined by the same controls, analysed on separate microtiter plates. All plates were processed by using optimal conditions.

3.2 Specificity of HSV-antigen in ELISA test

The specificity of HSV antigen was tested against antibody to herpesviruses group as followed :

- Herpes simplex virus antibody (Behringwerke, W. Germany)
- Varicella zoster virus antibody (Behringwerke, W. Germany)
- Cytomegalovirus antibody (Behringwerke, W. Germany)

Three commercial human sera were diluted in PBS-Tween albumin and processed by using optimal conditions.

4. Comparative study of HSV-antibody by ELISA (local-made), Commercial ELISA Kit, and IFA (local-made)

The developed ELISA technic was applied to study HSV-antibody in the serum of 92 subjects, compared to the commercial ELISA kit and IFA (local-made).

4.1 Procedure of ELISA (local-made)

Polystyrene microtiter plate (Nunc, Roskilde, Denmark) coated with 100 µl of HSV-antigen (80 µg/ml) in coating buffer, pH 9.6 was incubated at 4 °C overnight (18 hours) and washed three times before use. In the adjacent row, control antigen was processed in the same way. After washing, 50 µl of tested sera diluted 1:100 in PBS-Tween-albumin, were added to the wells in duplicate. The plate was incubated at 37 °C for 90 min, washed five times and reacted with 50 µl of antihuman-IgG peroxidase-conjugate diluted 1:2,400 in PBS-Tween-albumin, kept at 37 °C. After 30 min of incubation, the plate was washed five times and 100 µl of O-phenylenediamine substrate solution was added to each well, and further incubated at room temperature (in the dark) for 30 min. The reaction was stopped by adding 50 µl of 4 N H_2SO_4. The absorbance of the contents in each well was read at 492 nm in a ELISA-microtiter plate reader. To validate this test, positive- and negative-control serum were included in each plate (Fig.7).

4.1.1 Interpretation of results

In this test, the sera react with the control antigen, and the result of the nonspecific reaction must be substracted from the combined specific and nonspecific reaction given by the sera against the HSV-antigen. A positive reaction is determined by a

difference in optical density (O.D.) which is O.D. of HSV-antigen minus O.D. of control antigen must be equal or greater than 0.2.

4.2 Procedure of commercial ELISA kit

Test plate and all reagents must be brought to 20 ^{o} to 25 ^{o}C before use. The tested sera were diluted 1:44 in diluting buffer for detection of HSV antibodies (IgG). The ELISA-procedure of commercial kit was as follow.

- Introduced 0.15 ml of dilution buffer AP into each reaction well.

- Pipetted 0.05 ml in each case of the sample prediluted 1:11 into a reaction well coated with antigen and control antigen , respectively.

- Incubated the test plate for 1 h at 37 ^{o}C in moist chamber.

- Aspirated off the serum dilutions, and pipetted at least 0.2 ml of diluted washing solution into each well. After about 1 to 2 min, it was aspirated off, and repeated this washing process twice.

- Added 0.05 ml of the diluted enzyme-conjugate-solution to each well, and incubated the plate for 1 h at 37 ^{o}C in a moist chamber.

- Aspirated off the enzyme-conjugate, and washed the plate as above mention. Added 0.1 ml of substrate solution to each well, and incubated for 30 min

at 37 °C in a moist chamber.

- Stopped the enzyme reaction after the prescribed period by addition of 0.05 ml of stopping solution AP (2N NaOH).

- Evaluated the yellowish-green color reaction within one hour. Interpretation of results as in 4.1.1 .

4.3 Indirect immunofluorescent antibody technic (IFA) for HSV-antibody

HSV infected cells were fixed on the slide and incubated with tested serum diluted 1:10 in PBS. After which, immunostaining were carried out using fluorescein-isothiocyanate conjugated rabbit-antihuman IgG (Dako, Glostrup, Denmark) diluted 1:40 in PBS.

Grading system ; the reaction was positive when the infected cells in each field, approximately 10% exhibit a greenish-yellow fluorescence against the red background of the uninfected cells.

4.4 Statistical Analysis

Mean ($\bar{x}$) $= \Sigma \frac{x}{n}$

Standard deviation (SD) $= \sqrt{\Sigma \frac{(x-\bar{x})^2}{n-1}}$

% Coefficient of variation (CV) $= \frac{SD}{\bar{x}} \times 100$

% Sensitivity $= \frac{TP}{TP + FN} \times 100$

$$\% \text{ Specificity} = \frac{TN}{TN + FP} \times 100$$

$$\% \text{ Efficiency} = \frac{TP + TN}{\text{Total number of samples}} \times 100$$

$$\% \text{ Positive predictive value} = \frac{TP}{TP + FP} \times 100$$

$$\% \text{ Negative predictive value} = \frac{TN}{TN + FN} \times 100$$

TP = true positive, TN = true negative

FP = false positive, FN = false negative

5. Study of the stability of the ELISA antigen in various conditions.

The antigen was preserved in various conditions as follow :

5.1 Freezing : One milliliter of antigen was aseptically distributed in glass test tubes(12x75mm) and thightly closed with rubber stoppers. These antigens were kept at -20 °C until used.

5.2 Lyophilization : Five hundred microliters of antigen was aseptically distributed in each of the 15 lyophilized ampoules. The antigen in ampoules were pre-frozen with dry ice-acetone mixture and then immediately placed into the manifold of the lyophilizer. The suction pump pressure was set at 2×10^{-6} millibar. The full process of lyophilization took three hours by which time the antigen became powder. The ampoules were sealed by flame and kept at 4 °C.

5.3 Coating on solid-phase(microtiter plate) : The antigen with concentration of 80 µg/ml were coated on polystyrene microtiter plate(Nunc, Roskilde, Denmark) in the rows A, C, E, and G. The rows B, D, F, and H were coated with control antigen. This was done on 20 plates. The plates were then individually kept in sealed plastic bags at -20 °C.

5.4 Coating on solid-phase(microtiter plate) and washed before kept at -20 °C : The antigen were coated as described in 5.3 on 20 polystyrene microtiter plates. Prior to be kept individually in sealed plastic bags at -20 °C, the plates were then washed 3 times with PBS-Tween (see on page 35).

Sera : Forty sera composed of 32 positive and 8 negative sera were selected to be tested for antigen stability. The 40 sera were 0.05 ml individually aliquoted in 1 ml microcentrifuge tubes and kept at -20 °C.

When the scheduled time dued (1, 2, 3, and 6 months period) the 40 sera in aliquots were taken out and tested with each antigen preparations. Assay and interpretation of the results were that of described previously. Each aliquot was used only once.

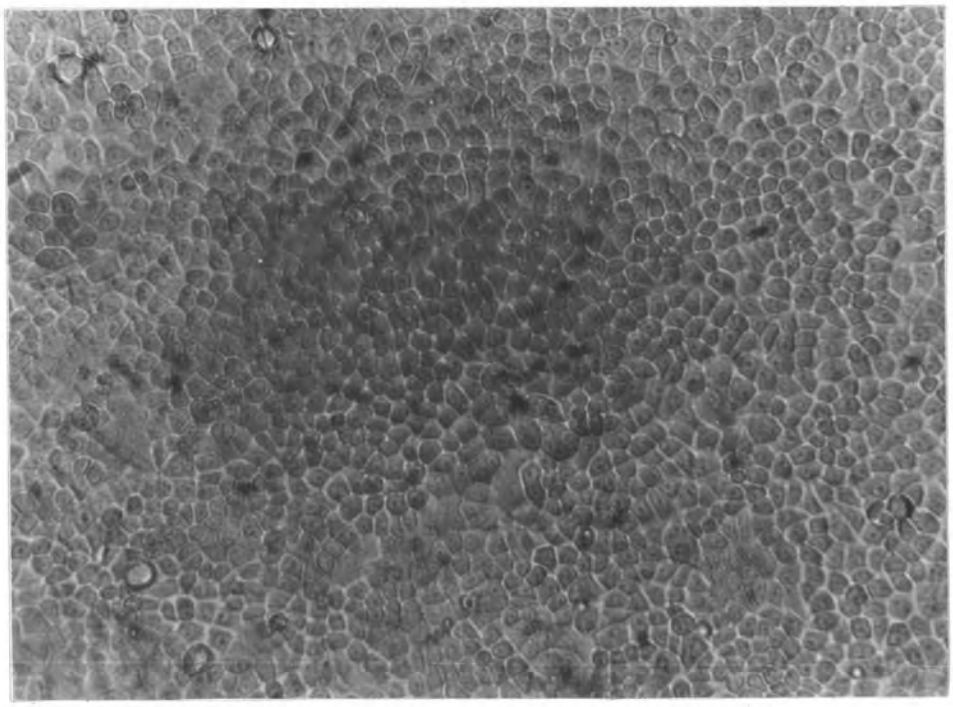

Fig. 1 Completed monolayer of HeLa cells

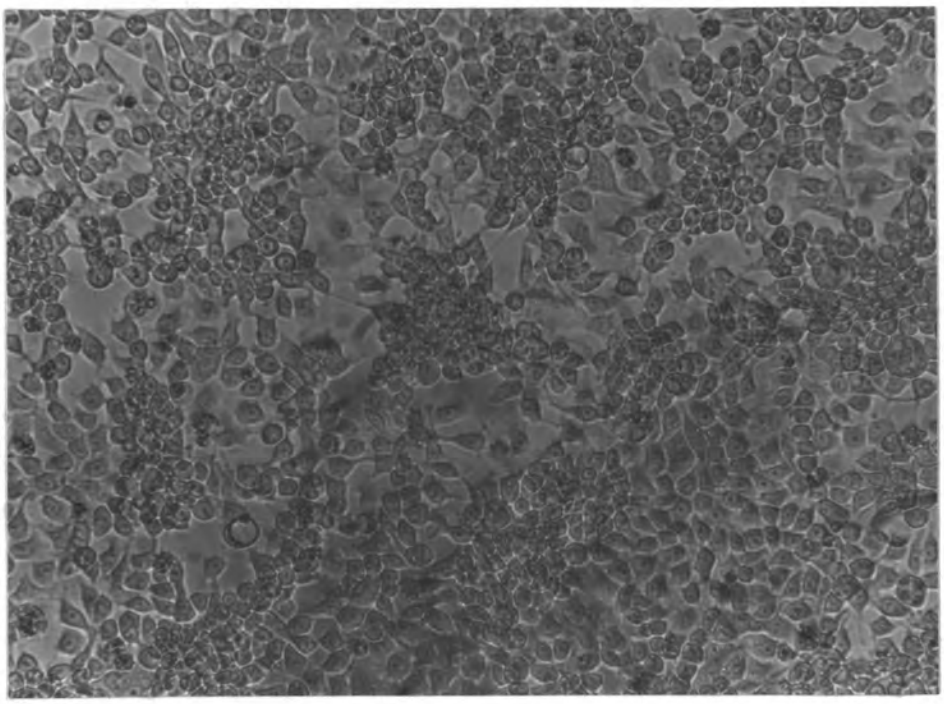

Fig. 2 Cytopathogenic effect of HSV-2 in HeLa cells

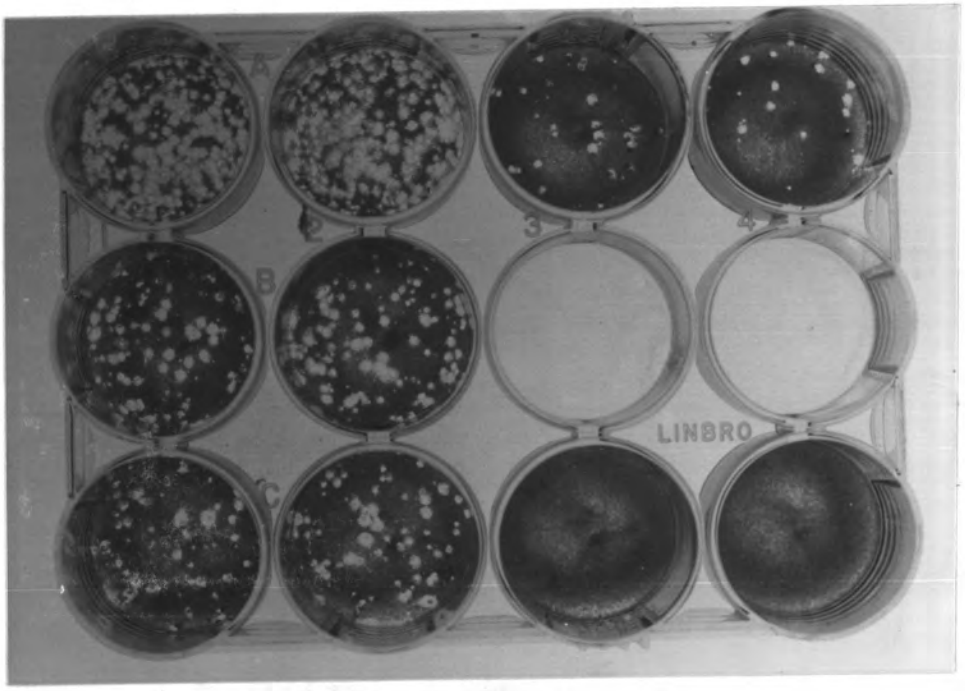

Fig. 3 Staining of plaque assay

Row A 1,2 : HSV-2 dilution 1:1,000

Row B 1,2 : HSV-2 dilution 1:10,000

Row C 1,2 : HSV-2 dilution 1:100,000

Row A 3,4 : HSV-2 dilution 1:1,000,000

Row C 3,4 : HeLa cell control

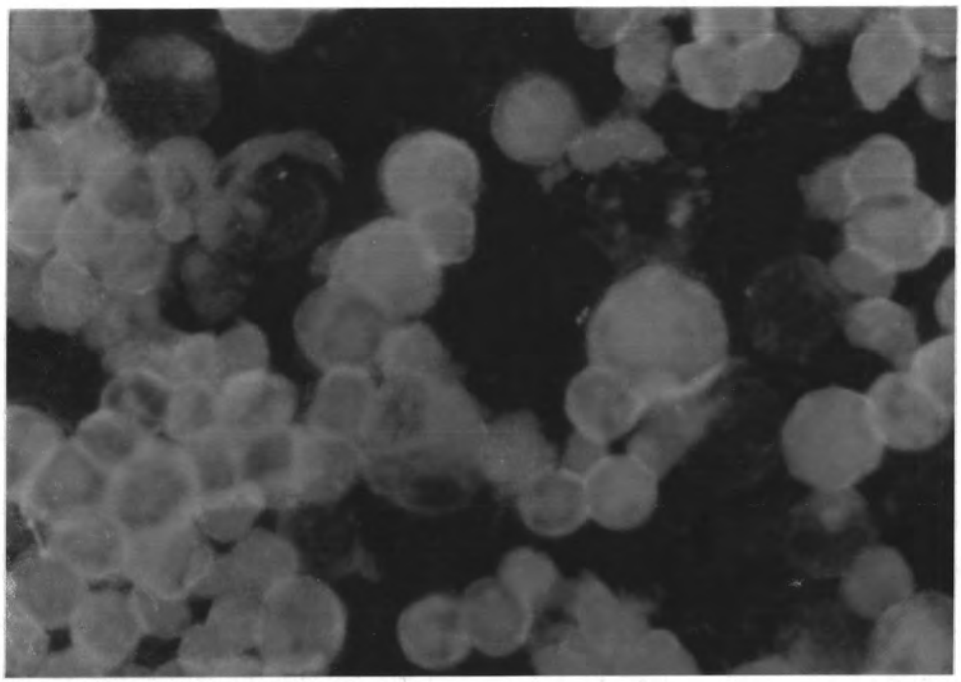

Fig. 4 A greenish-yellow fluorescence of HSV-2 infected cells

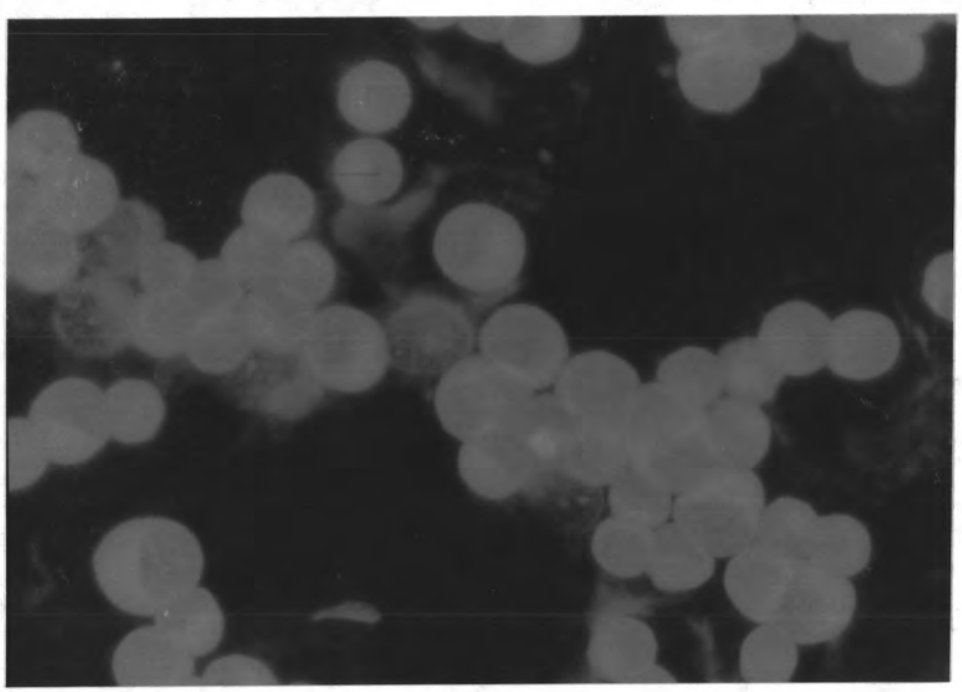

Fig. 5 The red background without fluorescence of uninfected cells

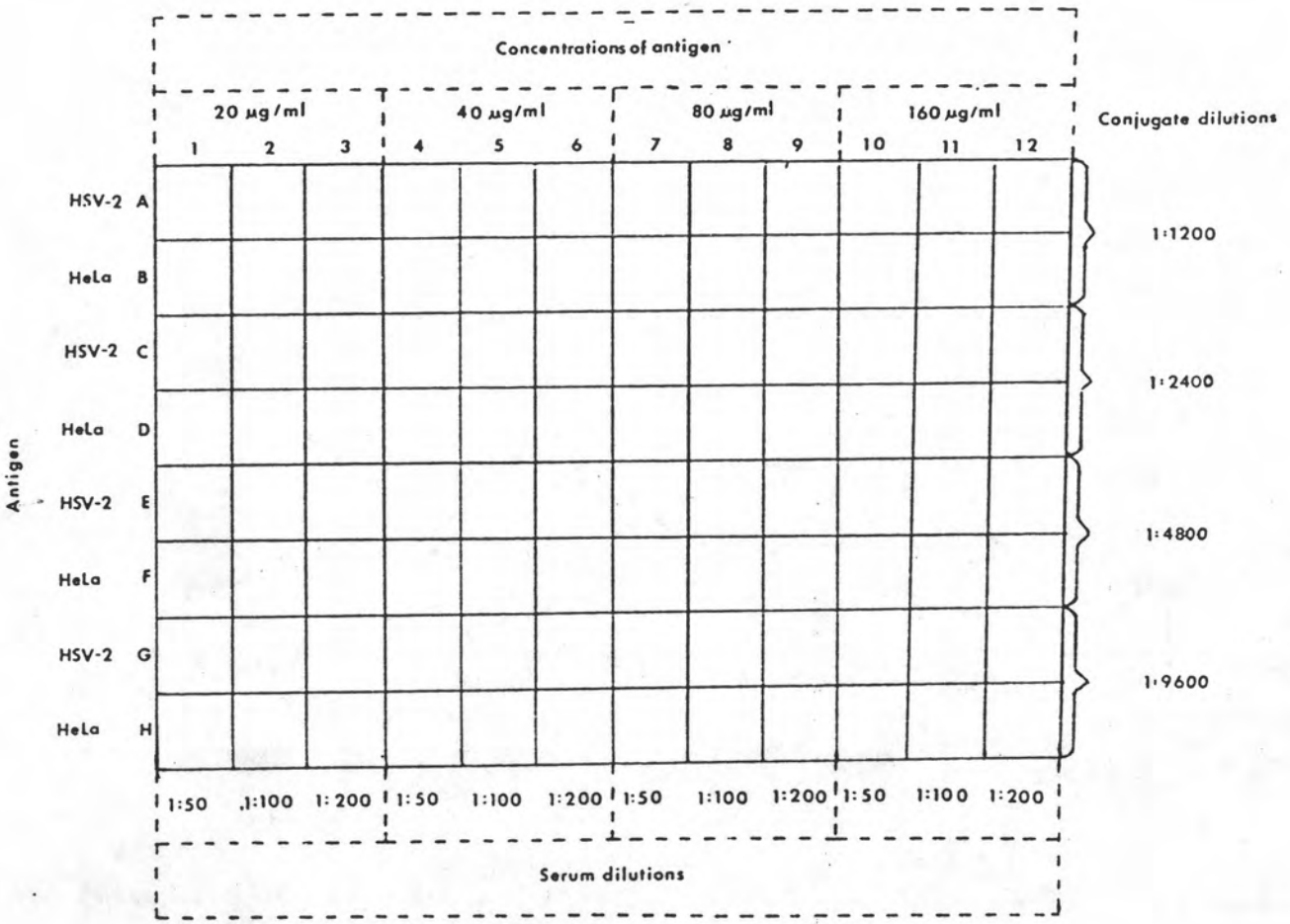

Fig. 6 Checker board titration

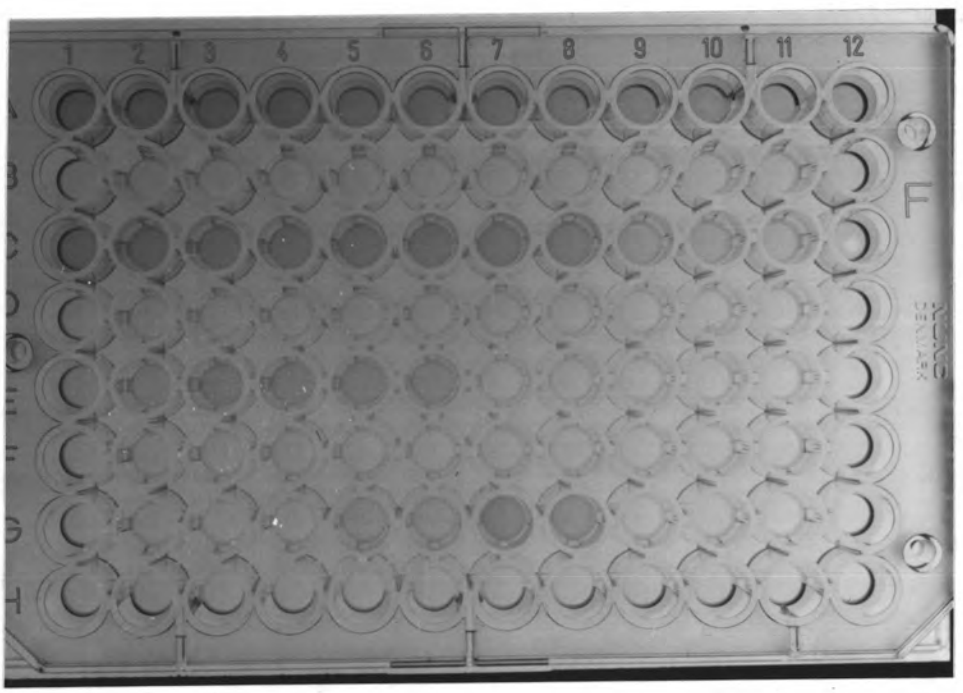

Fig. 7 Illustration of ELISA-plate, positive appears orange color while negative shows pale yellow color.

Row G/H 5,6 : weak positive control

Row G/H 7,8 : strong positive control

Row G/H 9,10 : negative control

Row G/H 11,12 : back ground

The other wells are tested sample.