CHAPTER II

MATERIALS AND METHODS

Materials

1. Collection of Specimens

The specimens used for this study were collected from two groups of women.

The first group comprised of 107 Thai pregnant women, attending to the Antenatal Care Unit of Chulalongkorn Hospital for their first prenatal visit during August 1984 to March 1985. These women were collected both cervical swab and venous blood specimens, three times each at the first, second and third trimester, respectively.

The second group comprised of 75 married women, attending to the National Cancer Institute for having routine examination. These women were obtaind only cervical swab specimens.

At the time of examination, each woman had questionaires; containing age, education, socioeconomic status, marital status, number of previous pregnancy and history of sexual transmitted diseases.

Subjects with symptoms and signs suggestive of genital Herpes were excluded because of the potential interference of HSV with isolation of CMV (Wentworth et al., 1973).

1.1 Cervical Swab Specimens

Sterile cotton swabs immersed in transport medium (as described on page 30) were used for sampling. To collect a specimen of cervical secretions, visualization of the cervix uteri by means of speculum examination was a prerequisite. Secretions were obtained by swirling the cotton swab on the external os of the cervical canal and dipped into 3 ml of transport medium. The specimens were kept at 4°C (in a thermos flask), no longer than 2 hours and then transported to the laboratory, kept at -70°C until testing (for virus study).

1.2 Venous Blood Specimens

A venous blood specimen collected aseptically (about 5 ml) was allowed to clot in a sterile test tube for 2 hours at room temperature. After clot retraction, preferably overnight at 4°C, the serum was separated and stored at -20°C until testing (for serological study). Do not freeze whole blood, this caused severe hemolysis and might render the specimens unusable for serological testing.

Sometimes, the venous blood specimens were separated by centrifugation at 2,000 RPM for 10 min. The serum was separated and stored at -20°C until testing.

2. Cell Cultures

For Human Cytomegalovirus (HCMV) isolation, the only cells that should be used in the diagnostic laboratory to grow HCMV are human fibroblast cells, and a readily available tissue source is neonatal foreskin. The HCMV will not replicate in

non-human cell line such as cultures of monkey cell e.g. Vero-cell (The African Green Monkey Kidney Cell).

2.1 Human Foreskin Fibroblast Cells

These fibroblast cells were obtained as primary cell cultures prepared from the newborn foreskin tissue (by circumcision) which was kindly supplied by the Neonatal Unit of Chulalongkorn Hospital, Bangkok. The establishment of explant culture was described on page 45.

2.2 Vero-cells

The Vero-cell culture, one kind of the continuous cell lines established from the African Green Monkey Kidney Cells, was kindly supplied by Research Division, AFRIMS, Bangkok.

3. Culture Media

3.1 Medium 199 (M 199)

Powder of M 199, containing Earle's Balanced Salts with L-glutamine without sodium bicarbonate in a package of one litre, was purchased from Flow Laboratories, Inc.

The preparation of 1x liquid medium:

3.1.1 Measure approximately 10% less deionized distilled water than desired total volume of medium (1 litre).

Water should be at room temperature (15-30°C).

Using a mixing container that is as close to the final volume as possible.

3.1.2 Add powdered M 199 to the water with gentle stirring. Do not heat the water.

3.1.3 Rinse out inside of package to remove all traces of powder.

3.1.4 Add additional water to bring medium to a desired volume. Stir until dissolved, and keep container closed until the medium is filtered.

3.1.5 The medium is sterilized immediately by membrane filtration.

3.1.6 Aseptically dispense into sterile containers.

3.1.7 Label and store at 4°C.

3.2 Minimum Essential Medium, Eagle (MEM)

Powder of MEM, containing Earle's salts with L-glutamine without sodium bicarbonate in a package of one litre, was purchased from GIBCO Laboratories.

The preparation of 1x liquid medium:

3.2.1 Measure approximately 10% less deionized distilled water than desired total volume of medium (1 litre).

Water should be at room temperature (15-30°C).

Using a mixing container that is as close to the final volume as possible.

3.2.2 Add powdered MEM to the water with gentle stirring. Do not heat the water.

3.2.3 Rinse out inside of packpage to remove all traces of powder.

3.2.4 Add additional water to bring medium to a desired volume. Stir until dissolved, and keep container closed until the medium is filtered.

3.2.5 The medium is sterilized immediately by membrane filtration.

3.2.6 Aseptically dispense into sterile containers.

3.2.7 Label and store at 4°C.

3.3 Transport Medium (with 35% sorbitol)

The medium was used to transport the cervical swab specimens. One hundred millilitres of the medium were prepared as follows:

Heat-inactivated FCS	2	ml
$NaHCO_3$, 1 M	1	ml
HEPES, 1 M	1	ml
Antibiotics:		
Gentamicin, 5,000 ug/ml	1	ml
Streptomycin, 20,000 ug/ml	1	ml
Fungizone, 500 ug/ml	1	ml

70% Sorbitol	50	ml
M 199 to	100	ml

The medium was sterilized by membrane filtration, three millilitres aliquots were aseptically dispensed into screw cap test tubes, and stored at 4°C.

3.4 Growth Medium (GM)

The medium was used for initiation of the cultures and was composed of ingredients which promoted rapid cellular proliferation. One hundred millilitres of the medium were aseptically prepared as follows:

Heat-inactivated FCS	10	ml
$NaHCO_3$, 1 M	1	ml
HEPES, 1 M	1	ml
5% L-glutamine	1	ml
Antibiotics:		
Penicillin, 20,000 u/ml	0.5	ml
Streptomycin, 20,000 ug/ml	0.5	ml
MEM, 1x to	100	ml

The medium should be stored at 4°C.

3.5 Maintenance Medium (MM)

The medium was intended to keep the cell cultures in a slow, steady state of metabolism during the time of virus replication, and it was less rich than GM in substances promoting

rapid cellular metabolism and proliferation. One hundred millilitres of the medium were aseptically prepared as follows:

Heat-inactivated FCS	2	ml
$NaHCO_3$, 1 M	1	ml
HEPES, 1 M	1	ml
5%, L-glutamine	1	ml
Antibiotics:		
Penicillin, 20,000 u/ml	0.5	ml
Streptomycin, 20,000 ug/ml	0.5	ml
MEM, 1x to	100	ml

The medium should be stored at 4°C.

4. Reagents

4.1 Reagents for Cell Cultures

4.1.1 Chick Embryo Extract, 50%

4.1.1.1 Chick embryos 9 to 10 days of age were harvested and placed in a sterile petridish where the eyes, beaks, legs and wings were removed and discarded. The remaining tissues were washed in a beaker containing MEM, 1x, then minced with scissors.

4.1.1.2 The tissue-mince was washed in MEM, 1x and then passed through a 10-ml syringe (without a needle) into a tissue grinder.

4.1.1.3 An equal volume of MEM, 1x, with gentamicin and fungizone for final concentration of 50 ug and 2 ug per millilitre of medium respectively, was added to the tissue grinder, minced for 1-2 min and allowed to stand at room temperature for 30 min.

4.1.1.4 The suspension was centrifuged at 2,000 RPM for 10 min and discarded the precipitate. The supernatant fluid was removed, freezing and thawing rapidly twice in dry ice-alcohol bath and 37°C water bath, then centrifuged at 2,000 RPM for 20 min and discarded the precipitate.

4.1.1.5 The supernatant fluid was removed, centrifuged at 18,000 RPM for 30 min and discarded the pracipitate. The supernatant fluid was removed, aseptically dispensed into the 1 ml-vials and stored at -20°C.

4.1.1.6 After thawing for use, the extract was clearified by centrifugation at 2,000 RPM for 10 min and diluted to 1:3 with MEM, 1x.

4.1.2 Chicken Plasma

The chicken were bled either from the wing vein or heart with a syring containing 1 ml of the anticoagulant (3.13% trisodium citrate solution) for each 10 ml of blood to be withdrawn. The anticoagulant-blood was centrifuged at 1,800 RPM for 10 min and separated the plasma which was sterilized by membrane filtration. One millilitre aliquots of plasma were aseptically dispensed into the vials and stored at -20°C.

4.1.3 3.13% Trisodium Citrate Solution

One litre of the anticoagulant solution was prepared as follows:

Trisodium Citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	31.3	g
Deionized distilled water to	1000	ml

The solution was sterilized by autoclave at 15 lb pressure for 15 min and stored at 4°C.

4.1.4 Fetal Calf Serum (FCS)

The desiccated fetal calf serum (DIFCO Laboratories, Detroit Michigan, USA) was rehydrated by aseptically adding 100 millilitres of deionized distilled water and gently twirl the bottle to facilitate solution of the contents. The rehydrated serum was heat-inactivated at 56°C (water bath) for 30 min and should be stored at -20°C.

4.1.5 Sorbitol, 70% Solution

The solution was used as an important component of transport medium to preserve the infectivity of CMV. The solution was prepared as follows:

D-Sorbitol (DIFCO Laboratories Detroit Michigan, USA)	70	g
Deionized distilled water to	100	ml

4.1.8 Phosphote Buffer Saline, 7.5 pH

The solution, free from Ca^{++} and Mg^{++}, was used for washing cell cultures before dispersion. One litre of the solution was prepared as follows:

NaCl	8.00	g
KCl	0.20	g
Na_2HPO_4 (anhydrous)	0.91	g
KH_2PO_4	0.12	g
Deionized distilled water to	1000	ml

The solution was sterilized by autoclave at 18 lb pressure for 35 min and stored at 4°C.

4.1.9 Trypsin Versene Solution (TV-Solution)

The solution was used as cell dispersing solution. Trypsin (E. Merk, Darmstadt) was a proteolytic enzyme which dispersed the cells before they were seriously damaged. This procedure used a combination of a proteolytic enzyme (trypsin) with a chelating agent (Versene or ethylenediaminetetra-acetic acid - EDTA) because TV-solution gave better cells dispersion and higher yields of viable cells than could be obtained with enzyme alone. One litre of the solution was prepared as follows:

NaCl	8.0	g
KCl	0.4	g
Glucose	1.0	g
Trypsin (E. Merk, Darmstadt)	1.5	g

The mixture was stirred until the sorbitol was dissolved, this could be aided by warming at 37°C (water bath). Then, the solution was sterilized by membrane filtration, and stored at 4°C.

4.1.6 HEPES Buffer, 1 M

The buffer was an organic buffer used to control the physiological pH range of the cell culture media. One molar solution of the HEPES buffer was prepared as follows:

HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)	235.3	g
Deionized distilled water to	1000	ml

The solution was sterilized by membrane filtration, and stored at 4°C. HEPES buffer was usually employed in cell culture media at concentrations of 20-25 mM.

4.1.7 Sodium Bicarbonate, 1 M

This was a buffering solution added, at the time of use, to balanced salt solution or cell culture media to provide proper buffering capacity. One molar solution of the sodium bicarbonate was prepared as follows:

$NaHCO_3$	84.01	g
Deionized distilled water to	1000	ml

The solution was sterilized by membrane filtration, and stored at 4°C.

The reagents were dissolved in approximately 800 millilitres of deionized distilled water, stirred for 1 hour (using magnetic bar and magnetic stirror). Then added as follows:

EDTA (Versene Fe-3 Fisher Scientific Company Chemical Manufacturing Division, New Jersey, USA)	0.20	g
$NaHCO_3$	0.58	g

The mixture was continuously stirred until the solution was dissolved (mixed well) and brought to the final volume of 1,000 millilitres by deionized distilled water. Then, the solution was sterilized by membrane filtration, and about five millilitres aliquots were aseptically dispensed into screw-cap test tubes. The solution should be stored at -20°C.

4.1.10 Antibiotics

The solutions were incorporated into the cell culture media for the cultivation of a variety of cell types, and also into the transport media for treating certain clinical specimens. Also, the solution in higher concentration was used for treating clinical specimens before inoculating them into cell cultures. The antibiotics used in this study were prepared as follows:

4.1.10.1 Gentamicin, 5,000 ug/ml

The stock solution was prepared by dissolving 80 mg/2 ml gentamicin sulfate (ANB Laboratories Co., Ltd., Bangkok, Thailand) in 14 millilitres of deionized distilled water to make concentration of 5,000 ug/ml and stored at -20°C.

4.1.10.2 Penicillin, 20,000 u/ml

The stock solution was prepared by dissolving 1,000,000 u/ml sodium penicillin G (Armed Forces Pharmaceutical Factory, Bangkok, Thailand) in 50 millilitres of deionized distilled water to make concentration of 20,000 u/ml and stored at -20°C.

4.1.10.3 Streptomycin, 20,000 ug/ml

The stock solution was prepared by dissolving 1 g/ml streptomycin sulfate (Armed Forces Pharmaceutical Factory, Bangkok, Thailand) in 50 millilitres of deionized distilled water to make concentration of 20,000 ug/ml and stored at -20°C.

4.1.10.4 Fungizone, 500 ug/ml

The stock solution was prepared by dissolving 50 mg/100ml Amphotericin B (E.R. Squibb & Sons, Inc., USA) in 100 millilitres of deionized distilled water to make concentration of 500 ug/ml and stored at -20°C.

4.2 Reagents for Indirect Immunofluorescent Antibody Test

4.2.1 Phosphate Buffer Saline, 0.01 M, 7.6 pH

This solution was used as diluent and washing buffer for IFA-Test. One litre of the stock buffer solution was prepared as follows:

Na_2HPO_4 (anhydrous)	13.36	g
$NaH_2PO_4 \cdot H_2O$	1.80	g
NaCl	85.00	g
Distilled Water to	1000	ml

The solution should be stored at 4°C.

Working PBS Solution

To prepare one litre of the working solution for use, combined 100 ml of stock buffer solution with 900 ml of distilled water. The solution should be checked and adjusted the pH to 7.6, then, stored at room temperature.

4.2.2 Acetone, AR Grade

Acetone (BDH Chemicals Ltd. Poole England) was universally acceptable fixative for viral antigens since these antigens were notoriously unstable and losed their antigenic characteristics when treated with many of the fixative such as ethanol, methanol, formalin (Riggs, 1979).

4.2.3 Antigen

The human foreskin fibroblast cell cultures, showing CPE after inoculated with cervical excretion of pregnant women, were used as testing samples.

The human foreskin fibroblast cell cultures; infected with CMV strain AD 169 which was kindly supplied by Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok; were used as control positive cells.

In addition, control negative cells were uninfected cells of human foreskin fibroblast cells cultures.

4.2.4 Antibody

The human serum with high-titered antibodies to CMV by Complement Fixation Test was selected from the patients attending at the Antenatal Care Unit of Chulalongkorn Hospital because the CMV-positive serum was not commercially available. The serum also was proven negative to HSV.

4.2.5 Conjugate

The 2 millilitres of rabbit anti-human IgG, specific for γ-chains, FITC-conjugated was purchased from DAKOPATTS, Denmark.

4.2.6. Counterstain

The counterstain was often desirable in order to quench non-specific green fluorescence and highten contrast between staining and background. Evans Blue, simple counterstain that provided deep red fluorescence, was employed in aqueous solution of about 0.01 to 0.005% concentration (Goldman, 1968). To prepare 1:30000 working solution, combined 0.1 ml of 1% stock solution with 30 ml of distilled water.

4.2.7. Buffered Glycerol Mounting Medium

To prepare 100 ml of PBS-buffered glycerol solution, used as mounting medium, combined 90 ml of glycerol solution (DIFCO Laboratories, Detroit Michigan, USA) with 10 ml of PBS 7.6 pH.

4.3 Reagents for Complement Fixation Test

4.3.1 Stock Veronal Buffer Saline (5x), 7.2 pH

One litre of the buffer, used as diluent for CFT, was prepared as follows:

Barbital (5,5 diethyl barbituric acid)	2.87	g
Sodium barbital (sodium 5,5 diethyl barbiturate)	1.00	g
NaCl	42.50	g
$MgCl_2 \cdot 6H_2O$	0.84	g
$CaCl_2$ (anhydrous)	0.14	g
Deionized distilled water to	1000	ml

The solution was sterilized by autoclave at 18 lb pressure for 35 min and should be stored at 4°C.

Working VBS solution

To prepare one litre of the working solution for use, poured 200 millilitres of 5x stock solution of VBS into a one-litre volumetric flask and brought to the final volume of 1000 millilitres of deionized distilled water. The solution should be stored at 4°C.

The working VBS solution must be made fresh on the day it is used.

4.3.2 **Hemolysin** (Amboceptor)

The 5 millilitres of Rabbit Anti-sheep red blood cells was purchased from Flow Laboratories, Inc. The hemolysin was titrated at the first time of CFT was performed, as described on page 59.

4.3.3 **Complement**

The guinea pig heart blood collected aseptically was allowed to clot in a sterile test tube for 1-2 hours at room temperature. After clot retraction, the serum was separated by centrifugation at 2,000 RPM for 10 min. Two millilitres aliquots of the complement were aseptically dispensed into screw cap test tubes and should be stored at -20°C. The complement should be titrated each time an antigen titration and diagnostic test were performed.

4.3.4 Antigens

4.3.4.1 CMV Antigen

The lyophilized antigen (Behringwerke AG, Marburg, W. Germany) was dissolved in 1 millilitre of deionized distilled water, and stored at -20°C.

4.3.4.2 CMV Negative Control Antigen

The lyophilized negative control antigen (Behringwerke AG, Marburg, W. Germany) was dissolved in 1 millilitre of deionized distilled water, and stored at -20°C.

4.3.5 Alsever's Solution (Modified)

One litre of the solution, used as anticoagulant solution, was prepared as follows:

Dextrose	20.50	g
Sodium Citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	8.00	g
Citric acid ($C_6H_8O_7$)	0.55	g
Sodium Chloride (NaCl)	4.20	g
Deionized distilled water to	1000	ml

The solution was sterilized by membrane filtration and stored at 4°C.

4.3.6 0.85% Normal Saline Solution (NSS)

One litre of the solution, used for washing the cells (sheep erythrocytes), was prepared as follows:

NaCl	8.5	g
Deionized distilled water	1000	ml

The solution was sterilized by autoclave at 18 lb pressure for 35 min and stored at room temperature.

4.3.7 Sheep Erythrocytes

Sheep blood, collected aseptically, was treated with an equal volume of Alsever's solution and stored at 4°C. The cells were aged for 5 days at 4-10°C before used. Properly collected blood of good quality would remain satisfactory for one month period if sterility was maintained.

Methods

1. Cell Cultures

1.1 Human Foreskin Fibroblast Cells

The primary human fibroblast cells were prepared from the foreskin tissues of one day old newborn infant. This was the explant culture employed plasma clot to aid attachment of tissue to the culture vessel. The newborn foreskin tissues obtained from circumcision was placed in the maintenance medium and kept cold while delivering to the laboratory. The tissues submitted to the laboratory for primary cell culture should be processed as soon as possible or within 24 hours after collection.

1.1.1 The Procedure for Preparing the Primary Explant Culture

1.1.1.1 Transfered the tissue to the sterile petridish and washed with maintenance medium. Dissected unwanted tissues, especially skin, out as much as possible, then cut the remaining tissue with scissor to about 1 mm cubes.

1.1.1.2 Dipped the tiny pieces of tissue into the chicken plasma for 1-5 min, then transfered by pasteur pipette to the 2-ounce tissue culture plastic flask, added one drop of 50% chick embryo extract over each piece of tissue and incubated at 37°C for 30 min.

1.1.1.3 After the incubation period, the plasma would clot and hold the tissue in place. Added approximately 5 ml of growth medium to the flask and tilted gently until all the fixed tissues were under the media level. Incubated at 37°C about 5-7 days, a substantial outgrowth of cells extending from the tissue would be observed (Figure 1). The chick embryo extract would be the source of the nutrients and stimulated migration out of the explant across the solid substrate (Freshney, 1983).

1.1.1.4 Replaced the medium weekly until the outgrowth had spread to cover the entire growth surface, at which point the cells should be passed (Figure 2).

1.1.2 **The Procedure of Monolayer Cells Subculture**

The first subculture represented an important transition for the culture. The need to subculture implied that the primary culture had increased to occupy all of the available substrate. Once a primary culture was subcultured (or passaged), it became known as a cell line (Freshney, 1983). The first subculture gave rise to a secondary culture and so on. The cell lines should also be given a code or designation. The procedure was described as follows:

1.1.2.1 Withdrew the medium and discarded.

1.1.2.2 Added approximately 5 ml of PBS free from Ca^{++} and Mg^{++} to the side of the culture flask opposite the confluent monolayer of primary human foreskin fibroblast cells, so as to avoid dislodging cells; rinsed the cells and discarded the rinse.

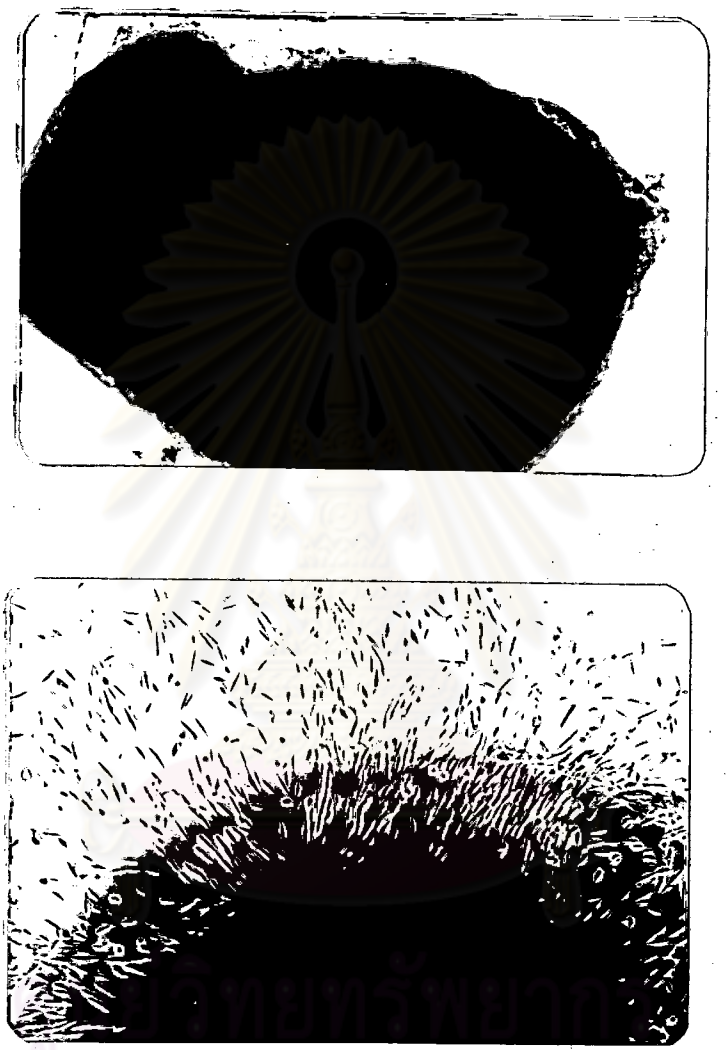


Figure 1 The primary explant from human (newborn) foreskin tissue. The fibroblast cells are seen migrating radially from the explant; unstained preparation. Magnification 100x

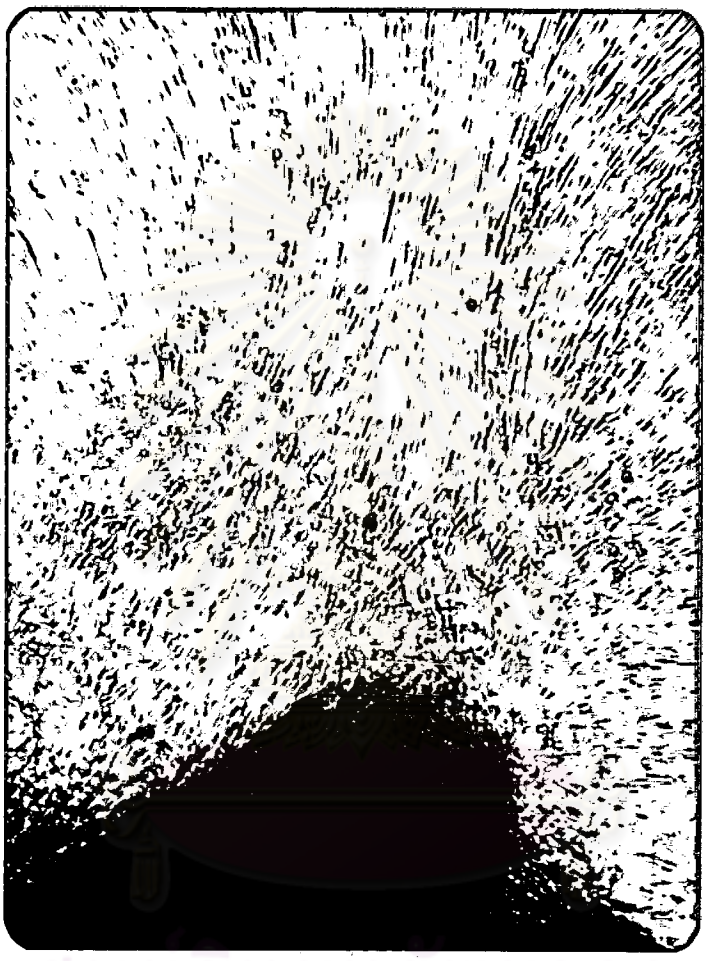


Figure 2 The confluent monolayer of primary human foreskin fibroblast cells, 5 weeks after the cultured preparation; unstained preparation. Magnification 100x

This step was designed to remove traces of serum which would inhibit the action of the TV-mixture (Freshney, 1983).

1.1.2.3 Added approximately 3 ml of TV-solution to the side of the culture flask opposite the cells. Turned the flask over, let TV-solution cover the monolayer completely, and left at room temperature until the cells rounded up (30-60 seconds). When the flask was tilted, the monolayer should slide down the surface.

1.1.2.4 Added an equal volume of maintenance medium (3 ml) to the flask and mixed gently, using a suckling-blowing action (Dispersed cells by repeated pipetting over the surface bearing the monolayer, pipetted the cell suspension up and down a few times, with the tip of the pipette resting on the bottom corner of the flask).

1.1.2.5 The trypsinized cell suspension was transfered into a centrifuge tube and centrifuged at 900 RPM for 5 min. Withdrew the supernatant and discarded, the cells were gently resuspended in growth medium at cell concentration 1 - 4 x 10^5 cells/ml and transfered to new bottles, incubated at 37°C until the cell sheets were confluent. At which point the cells should be passaged.

1.2 <u>Vero-cells</u>

The Vero continuous passage cell line of African Green Monkey Kidney Cells was employed for growth of a variety of viruses. The following procedure could be used for preparing cultures of this cell line.

1.2.1 Withdrew the medium and discarded.

1.2.2 Washed the cells with PBS free from Ca^{++} and Mg^{++} to remove traces of serum.

1.2.3 The cells in monolayer cultures were dispersed with a TV-Solution, left at room temperature until the cells rounded up and discarded the TV-Solution.

1.2.4 Added the growth medium to dilute the cells to contain 10^5 cells/ml, using a suckling-blowing action, and transfered to new bottles, incubated at 37°C until the cell sheets were coufluent. At which point the cells should be passaged.

1.3 Virus Isolation

According to the only cells that should be used to grow HCMV were human fibroblast cells, the isolation of HCMV from cervical swab specimens was described by the following procedure:

1.3.1 Diluted the human foreskin fibroblast cells, after the dispersing step, to the appropiate seeding concentration by adding the growth medium to the total volume required and distributing that among several screw cap test tubes (this usually planted with 10^5 cells/ml).

1.3.2 Incubated at 37°C until the confluent monolayer of fibroblast cells occurred, withdrew the medium and discarded, then inoculated approximately 0.1 ml of the treated cervical swab specimen into the culture tubes.

The cervical swab specimens were clearified by centrifugation at 2,000 RPM, 4°C, for 20 min and the supernatant was put aseptically into the sterile separate vials, kept at -70°C until testing.

1.3.3 During the incubation period which took about 45 min at 37°C, rolled the cultured tubes every 15 min. When the incubation period ended, discarded the inoculum and rinsed the cells with maintenance medium (or MEM 1x with penicillin and streptomycin) once and then discarded the rinse.

1.3.4 Then added 1 ml of the fresh maintenance medium and incubated at 37°C. The cultures were observed for cytopathic effect (CPE) every day for 4-6 weeks and the medium was replaced weekly.

The specimens containing Human Cytomegalovirus would show specific characteristic CPE within 3-4 days but usually in 1-2 weeks or longer, depending upon the amount of virus presented (Ho, 1982). If no CPE occured, it was considered negative. In addition of the test, the cell controls were also performed, by inoculating only the maintenance medium into the cell culture and incubated at 37°C.

1.4. **Virus Identification**

1.4.1 The clinical isolates were identified as CMV by their slowly developing CPE in tissue culture (Reynolds et al., 1979). Initially, foci of enlarged, rounded refractile cells would appear in the center rather than the peripheral of the cell monolayer. Often the affected cells had brownish refractile granules.

These foci would enlarge, coalesce, and might eventually destroy the monolayer, a process that might take 2-3 weeks. At times, foci of infection would enlarged, often with central degeneration, and satellite foci usually formed. As described above, the cytopathology of CMV was usually sufficiently characteristic for identification in the laboratory without further serologic confirmation (Ho, 1982).

1.4.2 Besides the typical characteristic CPE was considered as CMV identification, the failure of the virus to induce CPE in the non-human cells such as African Green Monkey Kidney Cells was also considered (Ahlfors, 1982, Chandler et al., 1985, Handsfield et al., 1985, Jordan et al., 1973, Lang and Kummer, 1972, Monif et al., 1972, Olson et al., 1970, Panjvani and Hanshaw, 1981, Pass et al., 1982, Peckham et al., 1983, Reynolds et al., 1973, Stagno et al., 1973, Stagno et al., 1975a and Stagno et al., 1975b).

According to the above references, the procedure used for identification of CMV wasdescribed as follows:

1.4.2.1 After the confluent monolayer of human foreskin fibroblast cells and also Vero-cells had formed, the cultures were ready to use, then withdrew the medium and discarded.

1.4.2.2 Approximately 0.1 ml of suspension of infected cells in isolated cultures that produced the characteristic CPE of CMV were inoculated into both human foreskin fibroblast cells and Vero-cells, respectively.

1.4.2.3 Incubated the cells at 37°C for 45 min, rolled the cultured tubes every 15 min. When the incubation period ended, discarded the inoculum and rinsed the cells with maintenance medium (or MEM 1x with penicillin and streptomycin) once.

1.4.2.4 Added 1 ml of fresh maintenance medium into the tube and incubated at 37°C. The cultures were observed for CPE every day for 4 weeks and the medium was replaced weekly.

CMV could produce CPE only in human foreskin fibroblast cells but not in Vero-cells.

1.4.3 In addition, CMV isolates showing CPE were also confirmed as HCMV by Indirect Immunofluorescent Antibody Test (IFA-Test), using human serum to contain antibodies to CMV (but not to HSV) and fluorescein isothiocyanate conjugated anti-human globulin (Fleisher et al., 1982, Plotkin and Huang, 1985, Rinaldo et al., 1977, Waner et al., 1977). The presence of fluorescence of infected cells at nuclear or cytoplasm, observed by microscopic examination with ultraviolet illumination, was considered positive result, indicating the presence of CMV in the specimens. The procedure of the IFA-Test modification was performed as previously described (Hanshaw et al., 1968, Starr and Friedman, 1980, Waner et al., 1980).

Procedural Outline

1.4.3.1 **Preparation of CMV Antigen on Slides**

The slide preparation was made from CMV-infected human foreskin fibroblast cells, as described by the following procedure.

Human foreskin fibroblast cells were seeded in tissue culture tubes. After the confluent monolayer of fibroblast cells occurred, the cells were infected with CMV strain AD 169 and CMV isolates.

When cell culture tubes showed 3^+ to 4^+ CPE, the cells were washed twice with PBS, 7.5 pH and were trypsinized by TV-solution. Then, added an equal volume of maintenance medium to the tube and the cells were sedimented by centrifugation at 900 RPM for 5 min (4°C).

The cells were washed once with PBS, 7.5 pH and centrifuged, the pellet was resuspended in a small volume of PBS, 7.6 pH (0.05 - 0.1 ml).

The cell suspension was dispensed as spots, 3 to 5 mm in diameter, on microscope slides. The spots were viewed microscopically under low power to assure that the spots had adequate cells then allowed to air dry at room temperature. Uninfected cell control spots were prepared in the same manner.

The air-dried cell slides were fixed in cold acetone for 10 min at room temperature and after fixation the slides might be used immediately in the staining procedure or stored at -20°C to -70°C until used.

1.4.3.2 **Titration of the Conjugate**

The fluorescein isothiocyanate (FITC) conjugated anti-human IgG should be assayed with a specific high-titered antiserum to determine the optimal dilution for use

prior to performing the test. The serial dilutions of conjugate and CMV-antibody positive serum were reacted with CMV infected and uninfected human foreskin fibroblast cells. The performance of the conjugate titration was described as follows.

Preparation of Serum Dilutions

CMV-antibody positive serum was diluted initially 1:5 in PBS 7.6 pH and further serial twofold dilutions in 7.6 pH were prepared.

Preparation of Conjugate Dilutions

The FITC conjugated anti-human IgG was serially twofold diluted in PBS 7.6 pH begining with the dilution of 1:10 and going beyond the range of activity.

Preparation of Counterstain Dilutions

The Evans Blue was diluted in distilled water to make the dilution of 1:30000, as the optimal dilution of the counterstain for use.

Setting Up the Conjugate Titration

The 20 ul of each serum dilution was individually applied to each smear of air-dried cells and allowed to react in the moist chamber at 37°C for 30 min.

After the incubation period, rinsed off the serum dilutions with PBS 7.6 pH and then washed in three changes of PBS 7.6 pH, 10 min each change.

Dried the smears under the dryer, then overlaid with 20 ul of each conjugate dilution and allowed to react in the moist chamber at 37°C for 30 min.

After the incubation period, rinsed off the conjugate dilutions with PBS 7.6 pH and then washed in three changes of PBS 7.6 pH, 15 min each change.

Immersed the slides in 1:30000 dilution of Evans Blue for 5 min and rinsed for 1 min with distilled water.

Dried the smears under the dryer, mounted in PBS-buffered glycerol solution with coverslip and examined under high-dry objective in a fluorescent microscope.

Determined the results according to the highest dilution producing 3^+ to 4^+ fluorescence of infected cells and no staining of uninfected cells with the highest dilution of antiserum was regarded as the optimal dilution of the conjugate.

1.4.3.3 **Performance of the IFA-Test**

Preparation of Serum Dilution

CMV-antibody positive serum was diluted in PBS 7.6 pH to make the dilution of 1:10, as the optimal dilution of antiserum for use.

Preparation of Conjugate Dilution

The FITC conjugated anti-human IgG was diluted in PBS 7.6 pH to make the dilution of 1:40, as the optimal dilution of conjugate for use.

Preparation of Counterstain Dilution

The Evans Blue was diluted in distilled water to make the dilution of 1:30000, as the optimal dilution of the counterstain for use.

Setting Up the IFA-Test

The 20 ul of 1:10 dilution of CMV-antibody positive serum was individually applied to each smear of air-dried infected and uninfected cells, and then allowed to react in the moist chamber at 37°C for 30 min.

After the incubation period, rinsed off the serum with PBS 7.6 pH and then washed in three change of PBS 7.6 pH, 10 min each change.

Dried the smears under the dryer, then overlaid with 20 ul of 1:40 dilution of FITC conjugated anti-human IgG and allowed to react in the moist chamber at 37°C for 30 min.

After the incubation period, rinsed off the conjugate with PBS 7.6 pH and then washed in three changes of PBS 7.6 pH, 15 min each change.

Immersed the slides in 1:30000. dilution of Evans Blue for 5 min and rinsed for 1 min with distilled water.

Dried the smears under the dryer, mounted in PBS-buffered glycerol solution with coverslip and examined under high-dry objective in a fluorescent microscope.

Determined the results according to the presence of typical fluorescence of infected cells, indicated the presence of CMV while no fluorescence of uninfected cells was produced.

Types of Controls

Positive Control: The positive control should be performed together with the IFA-Test by using the CMV strain AD 169 as antigen. The typical fluorescence was produced when the CMV antibody positive serum was applied to the CMV infected cells.

Negative Control: The negative control should be performed together with the IFA-Test by using the human foreskin fibroblast cells as antigen. The typical fluorescence was not produced when the CMV antibody positive serum was applied to the uninfected cells.

2. Serological Study

The Complement Fixation Test (CFT) for detecting antibodies to CMV was performed by using a microtiter technique as previously described (Reynolds et al., 1979). A titer of ⩾ 1:8 was considered positive, indicating the presence of CMV-CF-antibody.

Procedural Outline

2.1 Titration of the Hemolysin

The hemolysin titration was performed each time a new lot of hemolysin or sheep erythrocytes was used. Titration of hemolysin was performed as follows:

2.1.1 Preparation of 1:100 Hemolysin Solution

The 20 ul of hemolysin was mixed with 2.0 ml of VBS. The 1:100 hemolysin solution kept well at refrigerator temperature, but should be discarded if precipitate formed. Dilutions of hemolysin of 1:500 or greater were prepared by making further dilutions from 1:100 solution as shown in Table 1.

2.1.2 Preparation of 2% Sheep Erythrocytes Suspension

The cells were washed twice in 0.85% NSS and 2-3 times in VBS by certrifugation, at 2,000 RPM for 10 min. After the last washing, read the packed cells volume and diluted in VBS to 2% cell suspension. Stored at 4°C for no longer than 3 days.

Table 1 Preparation of Hemolysin Dilutions

Hemolysin Dilutions (ml)			VBS (ml)		Final Dilution of Hemolysin
1:100	= 0.5	plus	2.0	gives	1: 500
1:100	= 0.1	"	0.1	"	1:1000
1:100	= 0.1	"	1.5	"	1:1500
1:500	= 0.3	"	0.5	"	1:2000
1:500	= 0.2	"	0.8	"	1:2500
1:500	= 0.2	"	1.0	"	1:3000
1:500	" 0.2	"	1.2	"	1:3500

2.1.3 Preparation of the Sensitized Cells

Mixed an equal volume of each of the hemolysin dilutions (1:100 to 1:3500) with a 2% sheep erythrocytes suspension. The mixture was incubated in a 37°C water bath for 45 min with constant swirling of the contents. After the incubation period, the sheep erythrocytes became sensitized cells and were ready for use.

2.1.4 Preparation of the Complement Solution

The dilutions of complement were prepared by making further dilutions from undiluted complement as shown in Table 2.

2.1.5 Setting Up the Hemolysin Titration

2.1.5.1 Labeled eight wells of microtiter plate (U-Shape, Cat. No. 262170, NUNC, Denmark) with the hemolysin dilutions (1:100 to 1:3500) and with the complement dilutions as shown in Figure 3.

2.1.5.2 Added 25 ul of cold VBS to each well.

2.1.5.3 Added 25 ul of each of the following complement dilutions: 1:25, 1:30, 1:35, 1:40 ; and mixed well.

2.1.5.4 Added 50 ul of sensitized cells with each of the hemolysin dilutions to their appropiately labeled wells, and mixed well.

Table 2 Preparation of Complement Dilutions

Undiluted Complement (ul)		VBS (ml)		Final Dilution of Complement
20	plus	0.5	gives	1:25
20	"	0.6	"	1:30
20	"	0.7	"	1:35
20	"	0.8	"	1:40

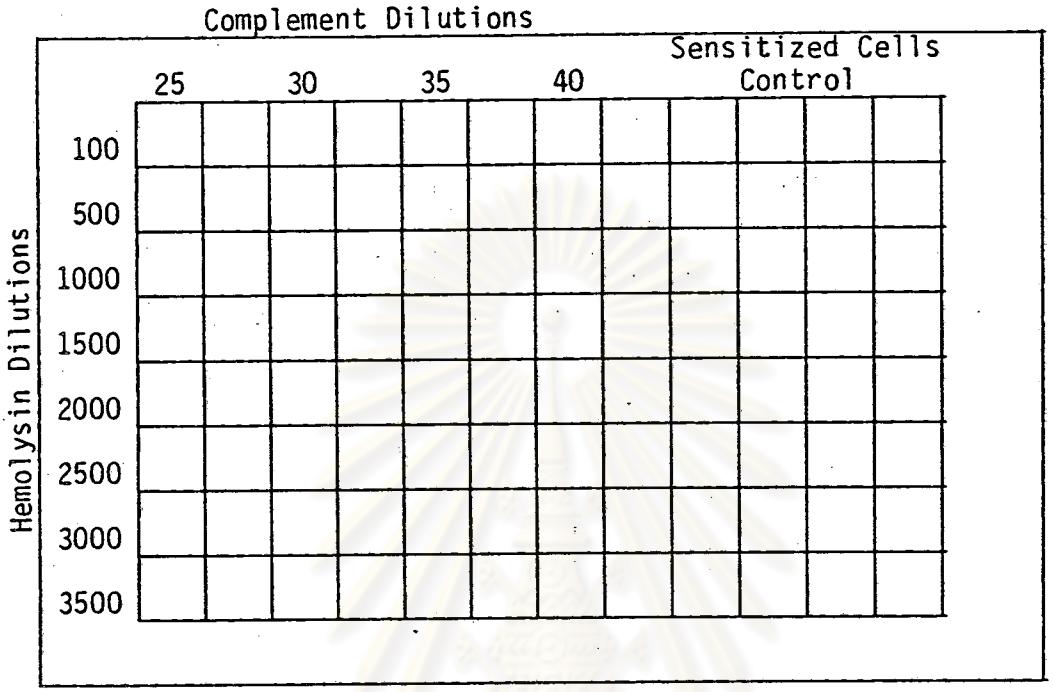


Figure 3 Setting Up the Hemolysin Titration

2.1.5.5 The plate was incubated at 37°C for 1 hour, and shaked after 30 min of incubation.

2.1.5.6 After the incubation period, recorded the results according to the highest dilution of hemolysin which showed complete hemolysis represented 1 unit. Used 2 units in the test.

2.2 Titration of the Complement

Complement was titrated each time the diagnostic tests were performed. Titration of complement was performed as follows:

2.2.1 Preparation of the Sensitized Cells

Mixed an equal volume of the optimal hemolysin dilution (1:1000) with a 2% sheep erythrocytes suspension. The mixture was incubated in a 37°C water bath for 45 min with constant swirling. After the incubation period, the sheep erythrocytes became sensitized cells and were ready for use.

2.2.2 Setting Up the Complement Titration

2.2.2.1 Labeled the wells of microtiter plate with the following complement dilutions; 1:2, 1:3, 1:4, 1:6, 1:8,...., 1:1536 ; and added the 25 ul of cold VBS to each well, excepted the second well (50 ul).

2.2.2.2 Added the 25 ul of the undiluted complement to the first and the second wells and made the series of dilutions in cold VBS from 1:2 and 1:3 dilutions of the complement, respectively.

2.2.2.3 Diluted the CMV-antigen to the 2 units test dilution, and added to all wells, 25 ul per well. Shaked the plate to mix well and incubated at 37°C for 1 hour.

2.2.2.4 After the incubation period, added 50 ul of sensilized cells to all wells, shaked the plate and incubated at 37°C for 30 min.

2.2.2.5 After the incubation period, recorded the results according to the highest dilution of the complement which showed complete hymolysis represented 1 unit. Used 2 units in the test.

2.2.2.6 To determine the good quality of the sensitized cells and the diluent, the sensitized cells control was performed by adding the sensitized cells to the VBS and showed no hemolysis.

2.3 Titration of the Antigen

The antigen titration was performed each time a new lot of antigen was used. Titration of antigen was performed by a checkerboard titration with serial dilutions of a specific high-titered antiserum against serial dilutions of antigen to determine the optimal dilution of antigen which gave fixation. The performance of antigen titration was described as follows:

2.3.1 Treatment of Serum

Prepared the 1:8 starting dilution of the antiserum and 1:8 negative serum in VBS, then inactivated in 56°C water bath for 30 min.

2.3.2 Preparation of Antigen Dilutions

Made a serial dilutions of antigen begining with 1:2 and going beyond the range of activity.

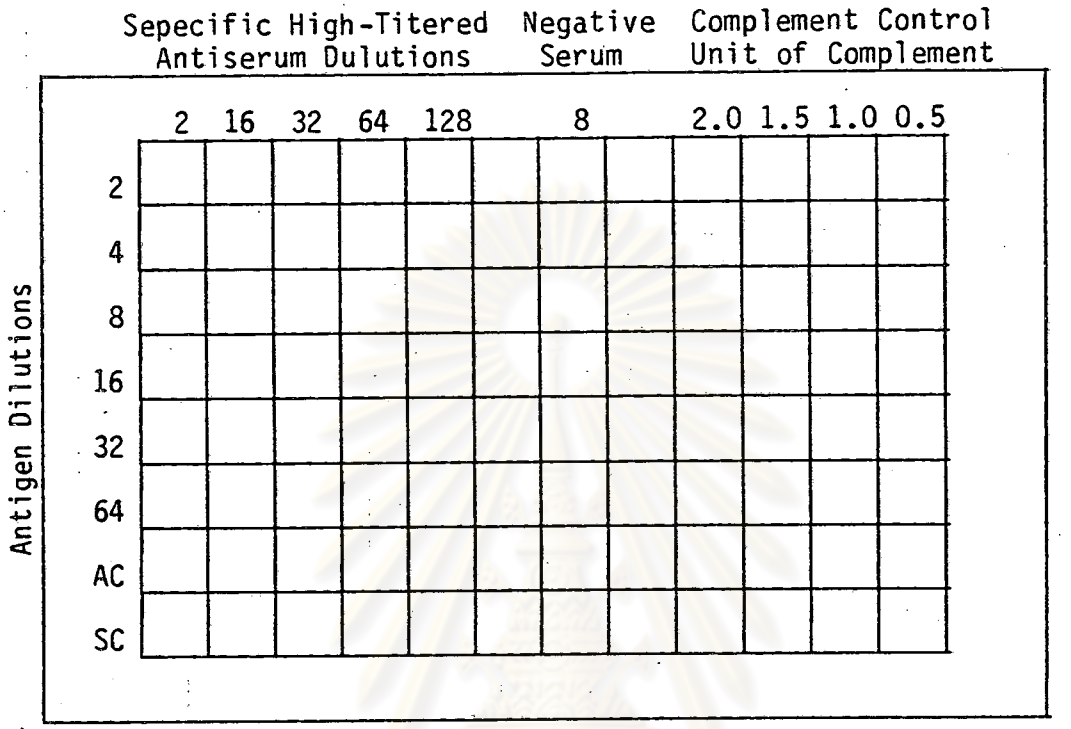
2.3.3 Setting up the Antigen Titration

2.3.3.1 Labeled the rows of wells on microtiter plate for the antigen titration as shown in Figure 4.

2.3.3.2 Prepared the further serial serum dilutions from the 1:8 inactivated antiserum, at least two dilutions beyond the known titer of the serum.

2.3.3.3 Added the 25 ul of each antigen dilution and the 25 ul of the complement containing 2 units to the antiserum and negative serum wells, starting with the highest dilution. Shaked the plate and incubated at 4°C overnight.

2.3.3.4 On the following day, warmed the plate for 15 min at room temperature; added the 50 ul of sensitized cells, mixed and then incubated at 37°C until the complement controls showed proper clearing (for 15-30 min).



AC = Antigen Control SC = Serum Control

Figure 4 Setting Up the Antigen Titration

2.3.3.5 Removed the plate from the incubator and hold it at 4°C until the unlysed cells had settled and the tests were ready to read. Recorded the results according to the highest dilution of antigen showing 3^+ or 4^+ fixation with the highest dilution of antiserum was generally regarded as 1 unit. Used 2 units of antigen in a volume of 25 ul.

2.3.4 Types of Controls Required for Antigen Titration

2.3.4.1 Antigen Control: Prepared the control antigen (uninfected tissue) to the same dilution used for the previous lot of antigen. Added 25 ul of control antigen to the appropiated wells instead of the antigen. Recorded the result according to the complete hemolysis.

2.3.4.2 Serum Control: The serum control used for testing the anticomplementary activity by adding 25 ul of VBS in the appropiated well instead of antigen. Recorded the result according to the complete hemolysis.

2.3.4.3 Complement Control: The complement control should be prepared together with the test (antigen titration) by diluting the complement in cold VBS to contain 2 units, and made the further serial dilution containing 1.5, 1.0, and 0.5 units from the 2 units complement dilution in the appropiated wells. Then, added 25 ul of each antigen dilution to the complement control wells.

When the complement controls showed the proper clearing, that was the wells containing 2.0 and 1.5 units of complement showed complete hemolysis, the wells containing

1.0 unit showed complete or nearly complete hemolysis, and the well containing 0.5 units of complement showed no hemolysis. If the wells containing 0.5 units showed hemolysis, an excess of complement was used in the test. If those containing 2.0 and 1.5 units did not show complete hemolysis, insufficient complement was used.

When the complement controls showed the proper degree of hemolysis, removed the plates from the incubator. If the complement controls were insufficiently cleared after the initial incubation period, continue incubation, examining the controls at frequent intervals during the succeeding 15 min. Removed the plates from the incubator when the controls showed the proper degree of hemolysis.

2.3.4.4 Sensitized Cells Control: To show the good quality of the sensitized cells and the diluent, the sensitized cells control should be performed by adding the sentitized cells to the VBS and showed no hemolysis.

2.4 Performance of the Diagnostic Test

2.4.1 Treatment of Serum

Because a non-specific reaction might be encountered with high concentrations of serum, an initial serum dilution of 1:4 in VBS was used in the test. The initial serum dilution was heat-inactivated in a 56°C water bath for 30 min to block the complementary activity.

If the serum showed anticomplementary activity, it could sometimes be blocked by adding the undiluted complement to the serum and incubated in a 37°C water bath for 30 min before inactivated in a 56°C water bath for 30 min.

2.4.2 Setting Up the Diagnostic Test

2.4.2.1 Labeled the rows of wells on microtiter plate for the diagnostic test as shown in Table 3.

2.4.2.2 Added 25 ul of cold VBS to dilution wells; added 25 ul of the initial serum dilution (1:4) to serum under test well, antigen control well, serum control well, and made the two-fold serum dilutions (for the serum under test) through wells.

2.4.2.3 Diluted CMV-Ag in cold VBS to contain 2 units, added 25 ul of CMV-Ag to the serum under test wells and complement control wells; and diluted CMV-negative control Ag to the same dilution, added 25 ul of CMV negative control Ag to the antigen control wells. The serum control wells were added 25 ul of VBS instead of antigen.

2.4.2.4 Diluted complement in cold VBS to contain 2 units, added 25 ul of the complement to all wells. Shaked the plate and incubated overnight at 4°C.

2.4.2.5 On the following day, warmed the plate for 15 min at room temperature, added 50 ul of sensitized cells, mixed and incubated at 37°C until the complement control showed proper clearing (for 15-30 min).

Table 3 Setting Up the Diagnostic Test

Wells	Serum (ul)	VBS (ul)	Ag (ul)	Control Ag (ul)	Complement (ul)		Sensitized Cell (ul)	
Serum under test	25	-	25	-	25	overnight incubation at 4°C followed by 15 min at room temperature	50	incubate at 37°C for 15-30 min
Serum control	25	25	-	-	25		50	
Antigen control	25	-	-	25	25		50	
Complement control								
Units 2.0	-	25	25	-	25		50	
1.5	-	25	25	-	25		50	
1.0	-	25	25	-	25		50	
0.5	-	25	25	-	25		50	
Sensitized cells								
Control	-	75	-	-	25		50	

2.4.2.6 Removed the plate from the incubator and hold it at 4°C until the unlysed cells had settled and the tests were ready to read. Recorded the results according to the following numerical values for the percent of hemolysis.

4 = no hemolysis

3 = 25% hemolysis

2 = 50% hemolysis

1 = 75% hemolysis

0 = complete hemolysis

The wells showing no hemolysis to 50% hemolysis considered as positive, estimating by the size of the nonhemolyzed cells button. In the absence of anticomplementary activity, positive reactions at serum dilution $\geqslant$ 1:8 indicated the presence of CMV-CF-antibody.

2.4.3 Types of Controls Required for the Diagnostic Test

2.4.3.1 Serum Control: The serum control used for testing the complementary activity by adding 25 ul of VBS in the appropiated wells instead of antigen. Recorded the result according to the complete hemolysis.

2.4.3.2 Antigen Control: Prepared the control antigen (uninfected tissue) to the same dilution as the antigen containing 2 units in the test. Added 25 ul of control antigen to the appropiated wells instead of the antigen. Recorded the result according to the complete hemolysis.

2.4.3.3 Complement control: The complement control should be prepared together with the test (diagnostic test) by diluting the complement in cold VBS to contain 2 units, and made the further serial dilutions containing 1.5, 1.0 and 0.5 units from the 2 units complement dilution in the appropiated wells. Then, added 25 ul of the antigen containing 2 units to the appropiated wells.

When the complement controls showed the proper clearing, the result was as previously described on page 69.

2.4.3.4 Sensitized Cells Control: To show the good quality of the sensitized cells and the diluent, the sensitized cells control should be performed by adding the sentitized cells to the VBS and showed no hemolysis.