CHAPTER IV

MATERIALS AND METHODS

Part I. Preparation of Cells and Stock Seeds Viruses

1. Cell Culture

Vero cell is a continuous cell line, initiating from the kidney of a normal adult African green monkey (*Cercopithecus aethiops*). These cells were obtained from the Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand.

Vero cells were grown in tissue culture flask at 37 °C atmosphere. Cell were grown in growth medium (GM) M199 (Earle's salt) with 10% Fetal bovine serum (GIBCO, BRL, U.S.A.), 0.01 M HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Sigma, U.S.A), and 100 units/ml penicilin G and 100 μg/ml streptomycin (GIBCO, BRL, U.S.A.). For maintenance medium (MM), the concentration of fetal bovine serum was reduced to 2%.

Dispersion of cell monolayers was performed by using trypsin-PBS (see Appendix II). The culture media was removed and the cell monolayers were washed twice with 5 ml phosphate buffered saline (PBS), pH 7.5 (see Appendix II) prewarmed to 37 °C. After discarding PBS one ml of trypsin-PBS was added and cells were incubated for one to two minutes at 37 °C. Then, the trypsin-PBS was discarded, the culture flask was gently knocked on palm until the cells were detached, GM was added. The cell monolayers were subcultured at three or four day intervals with a splitting ratio of 1:3.

2. Viruses

Standard HSV :

HSV-1 strain KOS and HSV-2 strain Baylor 186 were provided by Associate Professor Dr.Vimolmas Lipipun of the Department of Microbiology, Faculty of Pharmaceutical Science, Chulalongkorn University, Bangkok, Thailand.

Clinical Specimens:

A total of 121 specimens (obtained during January 1998- June 2002 from King Chulalongkorn Memorial Hospital) was divided into two groups according to site of infection, nongenital lesion and genital lesion.

Virus stock was prepared from Vero cell monolayers, confluent culture of Vero cells were infected with each clinical isolates of HSV at an approximate multiplicity of infection (MOI) 1.0 plaque forming unit per cell (PFU/cell). Adsorption of the virus was done at 37 °C for one hour, with occasional tilting of the plates, the unadsorbed virus was removed; the culture was washed once with 0.01 M PBS. MM media was added, and the virus infected cell was observed until 75 to 100 % of the cells show characteristic of CPE, usually 24 to 48 hours after infection. Then they were disrupted by being repeatedly frozen (at – 70 °C) and thawed (at 37 °C in water bath) for three times. The supernatant was collected by centrifugation at 4 °C, 2,200 rpm (IEC, U.S.A.) for 15 minutes. The supernatant fluid was distributed in small aliquots into vials and kept at -70 °C until use. All clinical isolates were assayed for the amount of infectious virus by Plaque titration.

3. Plaque Titration Assay

The CPE can be used to quantitate infectious virus particles by the plaque forming assay. HSV is one of the virus, which could from CPE, after cells infected with viruses. By using a semi-solid medium, a plaque is produced when a virus particle infects a cell, replicates, and then kills that cell. Surrounding cells are infected by the newly replicated viruses and they too are killed. The cells are then stained with a dye which stains only living cells. The death cells in the plaque appear as unstained areas on a colored background. However viruses which do not kill cells may not produce plaque. These plaque originate from a single infectious virus thus the titer of virus may be precisely estimated.

The virus was titrated in 96- well-plate (Nunclon, Denmark) and the titer was expressed as PFU/ml. In brief, the volume of 50 µl of each of the serial dilution of virus (10-fold) in MM was added in quadruplicate wells, followed by 50 µl of suspended Vero cell $3x10^4$ cells and incubated at 37 °C for three hours in order to complete cell settling then, 50 µl of overlay medium (0.8% gum tragacanth in GM) was applied. The medium was discarded after four to five days and the infected cells were stained with 1% crystal violet in 10% formaldehyde, for 20 minutes. The plate was washed, air-dried and the number of plaque was counted.

$$\text{PFU/ml} = \text{Dilution} \times \frac{P1 + P2 + \ldots Pn}{N} \times \frac{1}{V}$$

Where P = number of plaque counted in each well at this dilution.

N = number of wells

V = volume inoculated in the flasks (in milliliters)

Part II. Preparation of Viral DNAs

1. Preparation of Viral DNAs for HSV PCR Typing by QIAamp® DNA Mini Kit

DNA was extracted from culture cells by QIAamp® DNA Mini kit. The lysate buffering are adjusted to allow optimal of the QIAamp membrane before the sample is loaded onto the QIAamp spin column. DNA is adsorbed onto the QIAamp silica-gel membrane during a brief centrifugation or vacuum step. A confluent culture of Vero cells was infected with each isolate at MOI 1. The infected cells were scraped when they showed >75 % CPE, usually 16 to 18 hours after infection at 37 °C. The infected cells were washed twice with ice-cold PBS by centrifugation at 1,000 rpm for 10 minutes at 4 °C. The procedure of DNA extraction was followed the recommendation of the company. Briefly, the appropriate number of cells (approximately $5x10^6$ cells) were pelleted at 1,000 rpm for 5 minutes at 4 °C in 1.5 ml a microcentrifuge tube. The supernatant was removed out completely with taking care not to disturb the cell pellet. Cell pellet was resuspended in PBS to a final volume of 200 µl buffer AL, 20 µl Proteinese K and mixed by pulse-vortexing for 15 seconds, incubated at 56 °C for 10 minutes, followed by precipitation by addition ethanol (96-100%) 200 µl and mixed again by pulse-vortexing for 15 seconds, centrifuged at 8,000 rpm for 1 minute. The supernatant was transferred to QIAamp spin column. The filter was discarded. After centrifugation, the QIAamp column was washed with 500 µl buffer AW1 and 500 µl buffer AW2. After that, 100 µl distilled water was added incubated at room temperature for 1 minute and then centrifuged at 8,000 rpm for 1 minute. The eluate containing viral DNA was stored at -20 °C.

2. Preparation of Viral DNAs for Molecular Epidemiology Study of HSV by RFLP

A confluent culture of Vero cells were infected with each isolate at MOI of 1 to 5 PFU/cell. After an hour of viral adsorption at 37 °C, the unadsorbed virus was removed, the cells culture were washed once with PBS (see Appendix II), a MM (see Appendix II) was added and incubated at 37 °C. The infected cells were incubated until more than 75 % of the cell population showed CPE, usually 16-18 hours after infection at 37 °C. Then, HSV DNA was obtained free from host cellular DNA by Triton-NaCl extraction (42,65). Briefly, the infected cells monolayer was washed twice with ice-cold PBS and then scraped with cell scraper (Costar®, Mexico). The infected cells were pelleted in a 1.5 ml microcentrifuge tube by centrifugation at 1,000 rpm for 10 minutes at 4 °C. The cell pellet was resuspended in lysing solution (see Appendix II) at final concentration, suspension of infected cells approximately $1.5x10^7$ cell/ml and incubated at room temperature for 10 minutes with gentle mixing. 5 M NaCl (see Appendix II) was then added to a final concentration of 0.2 M and the preparation was centrifuged at 1,000 rpm for 10 minutes at 4 °C. The supernatant, containing the HSV DNA was carefully decanted and treated with RNase A final concentration 50 µg/ml (see Appendix II) and proteinase K final concentration 100 µg/ml (see Appendix II) for 2 hours each at 37 °C. Thereafter, HSV DNA was purified by phenol extraction, by mixing with an equal volume of phenol equilibrated (USB, U.S.A.), centrifuged at 8,000 rpm for 10 minutes (IEC, U.S.A.) at 4 °C. The upper aqueous layer was removed and transferred to a new 1.5 microcentrifuge tube. HSV DNA was further clarified by sequential extraction using an equal volume of phenol : chloroform : isoamyl (ultrapure) (USB, U.S.A.) 1 : 1 extraction two times, and the upper aqueous layer was removed and transferred to a new 1.5 microcentrifuge tube, followed by precipitation by addition of 0.1 volume of 3 M sodium acetate (see Appendix II) and two volumes of cold absolute ethanol were added. The mixture was kept at -70 °C for at least one hour. The DNA pellet was collected by centrifugation at 12,000 rpm for 15 minutes at 4 °C. The supernatant was poured off and the pellet was dried. TE buffer (see

Appendix II) was then added to dissolve the pellet. The extracted DNA was kept at -20 °C for further study.

3. Quantitation the Amount of DNA or RNA

Spectrophotometric Determination of DNA or RNA :

The amount of DNA was quantitated by using an ultraviolet spectrophotometer (SmartSpec ™ 3000, Bio-Rad, U.S.A.) at wavelengths of 260 nm and 280 nm (3). The reading at 260 nm allowed calculation of concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 µl/ml for ds DNA. The ratio between the OD at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimation of the purity of nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} ratio 1.8 and 2.0, respectively. If there is contamination with protein or phenol, the OD OD_{260}/OD_{280} ratio will be significantly less than these values.

Part III. Study Typing of HSV by (PCR)

1. PCR Typing of HSV

PCR typing of HSV was done as previously described by Jonson G *et al.* 2000 (341). The PCR amplification of HSV DNA region using consensus primers, which primers were designed to bracket a well conserved region in the DNA polymerase gene, based on an alignment of the DNA sequence of HSV was developed. Primer HSV P1 5' GTGGTGGACT TTGCCAGCC GTACCC 3' and HSV P2 5' TAAACATGGAGTCCGTGTCGCCGTAGATGA 3' were used to amplify HSV-1 and HSV-2. The characteristics of the expected amplicons for HSV length 532 base pairs. Each reaction was performed in PCR tube in a total volume of 50 µl, each reaction contained 5µl 10x PCR buffer (GIBCO, BRL, U.S.A.), 5 µl of 2.5 M $MgCl_2$ (Roche, Germany), 5 µl of deoxynucleotide triphosphate (dNTPs) (each dNTPs of 2mM), 2.5 µl of dimethyl sulfoxide (DMSO) (Sigma, U.S.A.), 37.5 pmol of each primer, 0.2 µl (two units) of taq polymerase (GIBCO, BRL, U.S.A.) and molecular grade double distilled water to a volume of 40 µl and 10 µl of template DNA was added. With the HSV P1 and HSV P2 primers pair the cycling parameters were initial preincubation at 95 °C for 12 minutes; then three cycles consisting of 95 °C for one minute, 60 °C for one minute, and 72 °C for three minutes; then 37 cycles of 95 °C for one minute, 55 ° C for 45 seconds, and 72 ° C for one minute; and then final incubation at 72 °C for three minutes. A 10 µl volume of each reaction mixture was subjected to electrophoresis.

2. Restriction Enzyme Digestion

Each reaction mixture in which amplicons were detected was subjected to digestion with the restriction enzyme *Bam*HI (Invitrogen, U.S.A.). The digestion mixture consisted of 10 µl of PCR product, 1.5 µl of appropriate enzyme buffer, 1 µl of enzyme, and 2.5 µl of double distilled water for a total volume of 15 µl. The reaction mixture was incubated at 37 °C for one hour.

3. Detection of DNA by Agarose Gel Electrophoresis

PCR product and digested products were analyzed by agarose gel electrophoresis (GE). This is a standard method used for separation, identification and purification of DNA fragments based on the principle of different DNA fragment mobility. 10 µl of the products were performed in horizontal gel electrophoresis by using 1.5% agarose gel (BRL, USA) in 0.5x Tris borate EDTA buffer (TBE) (see Appendix II). The electrophoresis was carried out at 90 volts for 50 minutes. The gel was stained in 1 µg/ml ethidium bromine and visualized under UV transilluminator and photographed.

4. Interpretation Species Identification by Restriction Enzyme Digestion of PCR Typing of HSV :

To simplify the practical procedure in the study, restriction enzyme digestions were carried out directly in the PCR mixtures. Although some restriction enzyme activities are very sensitive to differences in salt concentrations, the activity of the selected restriction enzyme, *Bam*HI, is fairly robust and partial digestions were rarely observed. The PCR product generated by these primers contained sufficient virus-specific DNA sequences for virus typing with restriction enzyme. Multiplex PCR was described in 1993 for the detection of HSV-1, HSV-2, CMV, EBV, and VZV. Methods for subsequent confirmation of the species of the virus detected with species-specific primers, and restriction enzyme analysis. The PCR assay for detection and species identification of HSV-1 and HSV-2, the detection of HSV in this assay is based on PCR with two primer pairs, followed by restriction enzyme analysis with *Bam*HI. Amplications obtained from the template DNAs described above were subjected to restriction endonuclease digestion with *Bam*HI. After digestion and electrophoresis, each viral DNA yielded a characteristic fragment pattern that corresponed to that predicted by DNA analysis. Even though HSV-1 and HSV-2 are cut at multiple sites by *Bam*HI, the larger fragments are clearly distinguishable and formed a pattern unique for each virus. The template DNAs region corresponding to the amplified fragments let us selected restriction endonuclease (*Bam*HI) suitable for the virus type identification, each viral DNA yielded a characteristic fragment pattern as expected. In case, which

a HSV virus was isolated by cell culture, the identification was determined by PCR assay followed by restriction fragment analysis (Table 1).

Table 2. Characteristic of amplicons as predicted by DNA sequencing

Virus	Length (bp)	% G+C content	BamHI site (position)[a]
HSV-1	532	66.2	No site
HSV-2	532	67.3	230
EBV	538	62.8	252
CMV	604	59.4	No site
HHV-8	526	54.6	No site
VZV	536	40.3	No site
HHV-6 variant A	533	44.5	246
HHV-6 variant B	533	44.7	246
HHV-7	533	37.9	No site

[a] the position of restriction site referred to the first base pair of the restriction site.

The band expected size for HSV digestion products was detected in the digestion reaction with *Bam*HI, but the fragment predicted from digestion of HSV-1 with *Bam*HI, was lacking. In case the presence of HSV-2 was ruled out by an additional digestion with *Bam*HI, which cut the HSV-2 amplicon (the *Bam*HI site is at nucleotide 230 bp) but does not cut the HSV-1 amplicon.

Part IV. Molecular Epidemiology Study of Genetic Variation of HSV by Restriction Fragment Length Polymorphism (RFLP)

1. Restriction Endonuclease Digestion and Agarose Gel Electrophoresis

Restriction endonuclease *Bam*HI, *Hin*dIII, *Eco*RI, and *Kpn*I (Invitrogen, U.S.A.) were used to analyse HSV genomic polymorphisms. HSV DNAs isolated from clinical specimens were cleaved with these REs. Reactions were performed in microcentrifuge tube. HSV DNAs containing approximately 3-5 µg of viral DNAs, sufficient REs was added to give a final concentration of 20 to 30 units, 1/10 volume of appropriate RE buffer for total volume of mixture reaction. The digestion mixture was incubated at 37 °C for three to four hours. At the end of the incubation time, digested products were separated by GE.

Separated by GE in 0.8 % agarose in TBE buffer, agarose was solubilized by heating in a microwave or on a hot plate. The melting agarose was allowed to cool to 60 °C before pouring the gel. The plastic gel chamber and gel former (14x20 cm) were used for electrophoresis. A well-forming comb was placed near one edge of the gel. The gel was allowed to harden until it became milky and opaque (approximately 30 minutes). After the comb and tape were removed, one liter of TBE was gently poured into the tank. The gel should be totally submerged in buffer, but not covered by more than 1 cm. Digested products were carefully loaded to individual wells of the agarose gel accompanied with a standard marker, DNA molecular weight marker IV (Roche, Germany) which contained 13 bands of DNA fragments that can be used to measure ds DNA fragments from 0.42 kbp to 19.3 kbp, λDNA-*Hin*dIII Digest which contained 8 fragments from 0.125 kbp to 23.130 kbp, and Φ X174-*Hae* III Digest which contained 10 fragments from 0.072 kbp to 1.353 kbp. Gel was electrophoresed at 60 volts for 15 hours for *Bam*HI and 17 hours for *Hin*dIII, *Eco*RI, and *Kpn*I, in running buffer.

2. Photography of DNA Fragments

After electrophoresis was completed, the gel was stained in ethidium bromide solution (See Appendix II), at a final concentration 1 μg/ml for 30 minutes and destained with distilled water for 5 minutes. The gel was photographed under UV light produced by a UV transilluminator (Chemi Doc, Bio-Rad, U.S.A.).